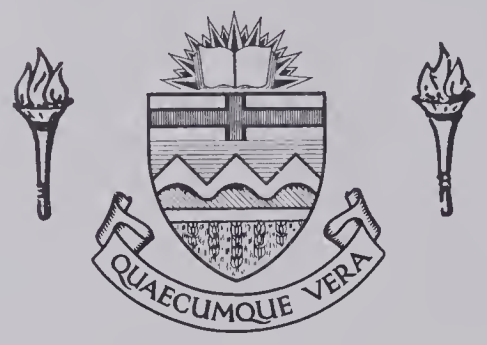

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GAS-LIQUID CHROMATOGRAPHY OF URINARY STEROIDS

by

© ROBERT DAVID BAYNTON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "GAS-LIQUID CHROMATOGRAPHY OF URINARY STEROIDS", submitted by Robert David Baynton in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

The diagnostic value of urinary estriol, pregnanediol and pregnanolone measurements in many complications of pregnancy is well established. However, it has been customary to determine these steroids by separate procedures. In order to obtain such data more quickly, a gas-liquid chromatographic method has been developed that permits simultaneous determination of the three steroids from a single urine specimen. A study has been made to establish optimum conditions of hydrolysis, derivatization and the final gas-liquid chromatographic step. A diagnostically useful laboratory method has resulted.

A gas-liquid chromatographic method for the determination of non-pregnancy urinary pregnanediol and pregnanetriol has also been developed. The applicability of this method to follow changes in these steroid levels during the menstrual cycle has been demonstrated. Another use of pregnanetriol analysis was demonstrated in a case of adrenogenital syndrome in which extremely elevated pregnanetriol levels were found.

Although the knowledge of non-pregnancy urinary estriol levels is certainly of diagnostic aid in various ovarian and adrenal clinical problems, the use of estriol analysis has been discouraged by the length and complexity of the

available methods. A simplified gas-liquid chromatographic method involving enzyme hydrolysis, organic solvent extraction, alkali treatment, organic solvent extraction of the trimethylsilyl ether derivative of estriol and programmed temperature gas-liquid chromatography was developed. Due to the speed and relative simplicity of this method, it readily lends itself for investigation of various clinical problems involving abnormal estrogen metabolism.

ACKNOWLEDGEMENTS

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This work was carried out in the Department of Clinical Laboratories, University of Alberta Hospital, and appreciation is extended to Dr. R.E. Bell, Director, for use of these facilities.

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LIST OF STEROID ABBREVIATIONS

Estradiol	1,3,5(10)-Estratrien-3β,17β-diol
Estriol	1,3,5(10)-Estratrien-3β,16α,17β-triol
Estrone	1,3,5(10)-Estratrien-3-ol-17-one
Pregnanediol	5β-Pregnane-3α,20α-diol
Pregnanetriol	5β-Pregnane-3α,17α,20α-triol
Pregnanolone	5β-Pregnane-3β-ol-20-one

I. ESTROGENS AND PROGESTERONES

A. METABOLISM

Estrogens (3-hydroxy-Δ1,3,5 estratriens) are produced in the female by the ovary, adrenal cortex and the placenta (1). They are responsible for normal growth and development of the reproductive organs and secondary sex characteristics. In the first half of the menstrual cycle, they stimulate regeneration of the endometrium and render it sensitive to progesterone, which acts during the second half of the menstrual cycle. The sudden fall in estrogen level at the end of the cycle initiates menstruation. Estrogens suppress the production of the pituitary hormone, Follicle Stimulating Hormone (FSH) which initially starts development of the follicle. In males, estrogens originate from the testes and the adrenal cortex (1). They have no significant effect when produced in normal amounts.

Several estrogens have been isolated, but only estrone, estradiol and estriol levels, the "classical estrogens", have been clearly related to specific clinical problems

Estradiol is the most potent of the three estrogens, followed by estrone and then estriol, which has little biological activity (2). According to Fishman *et al* (3) the principle estrogenic hormone in the ovary is estradiol, whereas estrone is probably the circulating estrogenic

hormone. Estriol is the main estrogen found in the placenta and urine of pregnant women. It is believed that the large estrogen excretion during pregnancy, of which 92 - 95% is estriol, is related to the amount and status of the trophoblastic tissue present in the placenta. Cassmer has demonstrated that urinary estrogen excretion in pregnancy is related to the presence of a living fetus (4).

Figure I shows the two major routes of biological synthesis which lead to the production of estriol. In a non-pregnant state, estriol is mainly produced via the progesterone, androstenedione and estradiol pathway. However, during pregnancy, the predominant pathway of estriol synthesis is through fetal adrenal metabolism of hydroxypregnenolone and hydroxydehydroepiandrosterone. Hydroxydehydroepiandrosterone is then converted to estriol in the placenta by aromatization of the steroid A ring and subsequent hydroxylation at the C-17 position of the steroid nucleus.

In the liver, estradiol is also converted to estrone and estriol. Here the estrogens are inactivated, conjugated to glucuronic or sulfuric acid and excreted in the urine and feces.

Progesterone (Δ-pregnene-3,20-dione), as shown in Figure II, is formed from cholesterol. The synthesis of progesterone occurs in the corpus luteum, the adrenal

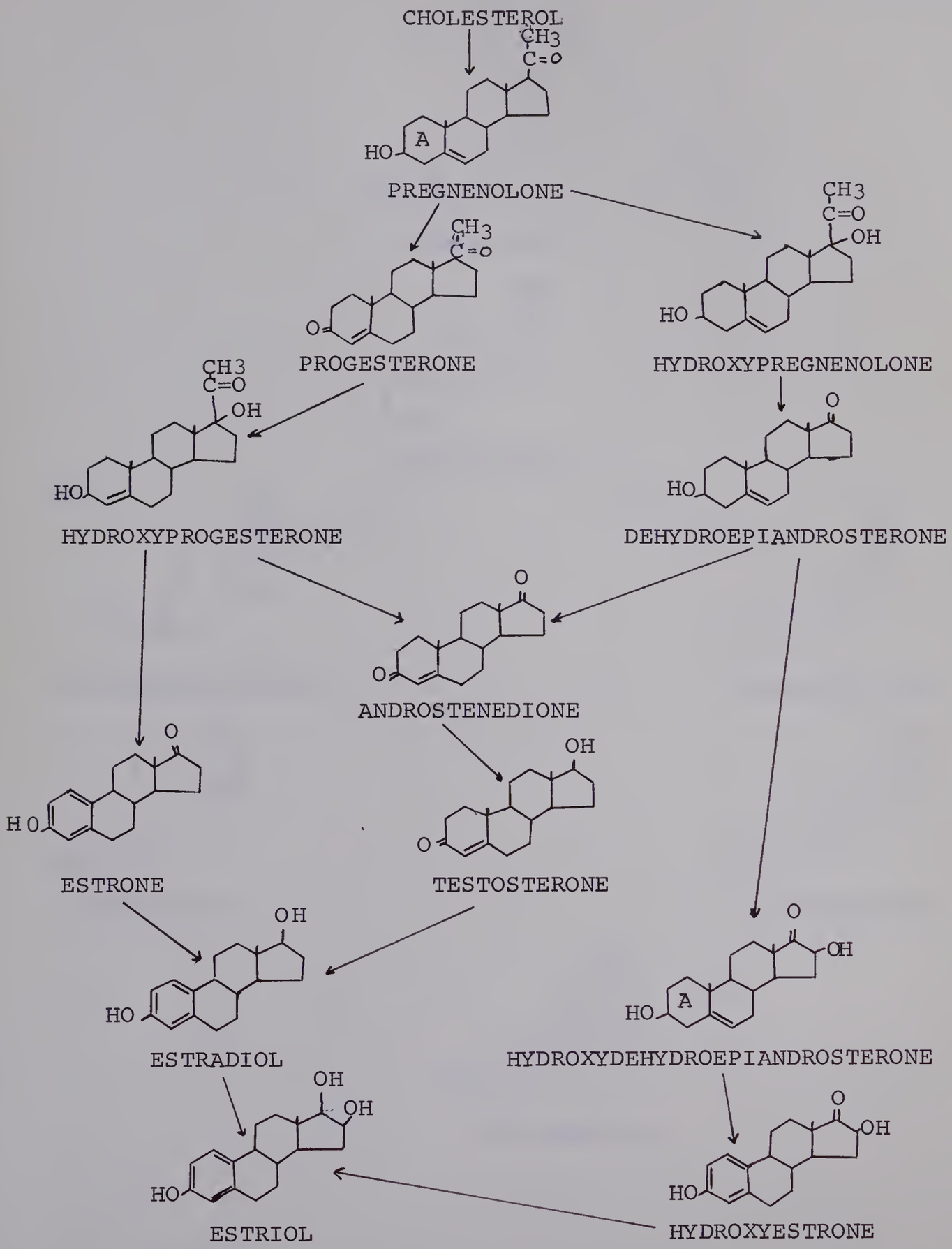

FIGURE I. Metabolism of Estrogens

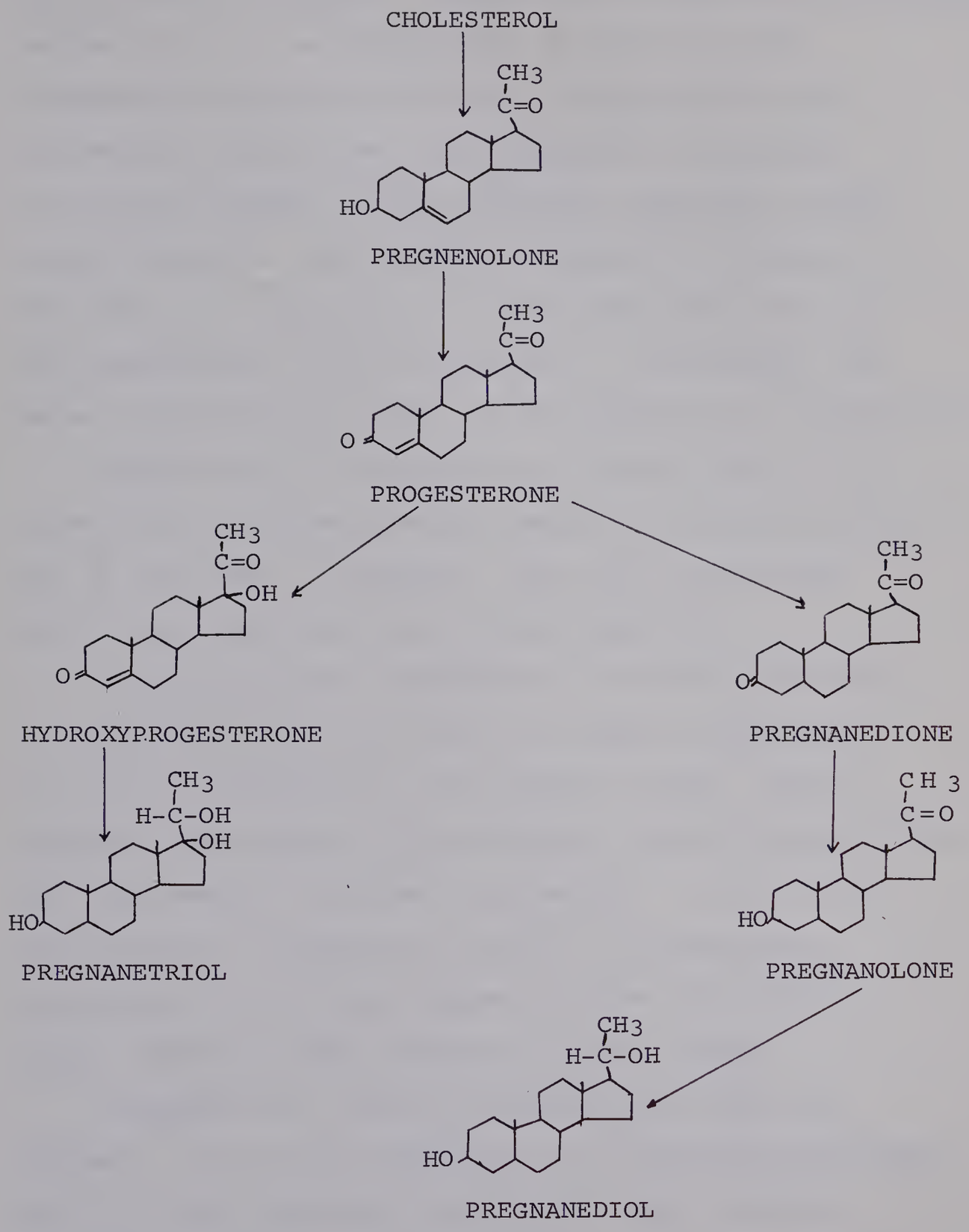

FIGURE II. Metabolism of Progesterone

cortex and the placenta. Progesterone converts the proliferative endometrium to a secretory phase at which time the progesterone excretion is higher. Progesterone also suppresses ovulation and the production of pituitary luteinizing hormone, which originally stimulated corpus luteum formation. Its overall function is to prepare the uterus for implantation of the fertilized ovum and for maintaining it during pregnancy. Progesterone also helps prepare breast tissue for the function of lactation (5).

Progesterone is metabolized so rapidly that it is found only in extremely minute amounts in blood and not at all in the urine. Therefore, assay for progesterone would seem impractical for clinical purposes.

Pregnanolone and pregnanediol are inert metabolites of progesterone and are formed, as shown in Figure II, by various hydroxylation and reduction steps. During pregnancy the output of progesterone, and thus pregnanolone and pregnanediol, increase considerably. In the first three months of pregnancy, the site of progesterone production is the corpus luteum and then the placenta begins secretion, soon becoming the sole source.

Pregnanetriol, found to originate only from the adrenals, is an inert metabolite of 17-hydroxyprogesterone, which in turn originates from progesterone. Little variation of pregnanetriol excretion occurs throughout life and levels of excretion do not vary significantly

between females and males.

B. CLINICAL SIGNIFICANCE

1. In normal males and females

During the menstrual cycle, the amounts of the three estrogens rise and fall together, with a maximum production during ovulation and another maximum during the luteal phase. The first peak is due to the Graffian follicle increasing to its maximum size. Rupturing of the follicle at ovulation causes a temporary arrest in estrogen production. Growth of the corpus luteum leads to another maximum excretion peak, the luteal peak, and if fertilization does not occur, regression of the corpus luteum causes estrogen production to drop.

Total urinary estrogen excretion of normal females varys from 4 - 65 mcg./day, depending on the menstrual cycle. During post-menopausal life, total estrogen excretion drops to 3 - 8 mcg./day (6).

In normal males, the source of estrogen production is the adrenal glands and the testes. Little change in estrogen excretion is observed and normal values for total estrogens range from 4 - 25 mcg./day. A slight increase has been noted in older men, but no significance has been attached to this finding.

In a normal ovulating female, increased production of progesterone begins about twelve days before the onset

of menstruation (a day or two after ovulation). The source of progesterone production is the corpus luteum. A peak is reached about five days later and if fertilization is not accomplished, the corpus luteum degenerates to the corpus albicans. The production of progesterone drops to its basal level, its source being solely the adrenals.

The normal range of pregnanediol excretion during the proliferative phase is 0.5 - 1.5 mg./day. During the luteal phase, it increases to 2 - 7 mg./day. In post-menopausal women, excretion ranges from 0.2 - 1.0 mg./day (6).

Pregnanetriol excretion is a function of adrenal activity which in turn depends on Adrenocorticotropic Hormone (ACTH) secretion by the anterior pituitary. Hydrocortisone, which is produced by the adrenal cortex, regulates the production of ACTH by a negative feedback mechanism. If this control mechanism is interrupted, as in congenital adrenal hyperplasia, ACTH will continue to stimulate the adrenal cortex and the result is an excess production of adrenocortical hormones, including pregnanetriol. Increased pregnanetriol excretion is considered to be one of the most consistent findings in cases of congenital adrenal hyperplasia due to deficiency of 21 -hydroxylase.

In normal adults, the range for urinary pregnanetriol excretion is from 0.2 - 4.0 mg./day. Excretion in children is usually less than 0.5 mg./day.

2. In pregnancy, normal and complicated

During pregnancy, dramatic changes occur in estrogen production. Although both estrone and estradiol levels increase considerably, estriol production far surpasses them. Brown (7) found that the ratio of estriol:estradiol: estrone changed from 3:3:1 in non-pregnancy to 30:2:1 in pregnancy. In a study of urinary estrogen metabolite excretion during pregnancy, Larsen and Engstrom (8) have shown that no furthur clinical information was obtained from measuring estradiol and estrone excretion levels, and estriol estimation was sufficient. Thus in the work described here, only the measurement of estriol was emphasized.

The production of estriol during pregnancy depends on both the placenta and the fetus, the two functioning as a biosynthetic unit. The fetus' adrenal glands produce hydroxydehydroepiandrosterone which passes via the umbilical artery into the placenta (9,10) and is converted into estriol.

As an estimate of fetal viability, measurement of estriol excretion during the early part of pregnancy is of little value. However, when the adrenals of the fetal component of the feto-placental unit become more active, estriol levels reflect fetal viability (11). During the first four months of pregnancy, the urinary estriol represents estrogen production by the trophoblast and ovary

while in the last five months of pregnancy, the effects of fetal metabolism become increasingly more important (12). Estriol excretion increases from a level of 0.1 mg./day in the first trimester of pregnancy to levels of 20.0 mg./day, and even up to 50.0 mg./day, in the latter part of the third trimester (7). Maternal estriol excretion has been found to correlate well with fetal size, which in turn is closely associated with the size of the fetal adrenal glands (11).

In certain complications of pregnancy, urinary estriol excretion provides a useful index of fetal viability. For example, Frandsen and Stakeman (13) studied sixty-six cases of abnormal pregnancies which involved a variety of clinical problems. In all but one, the fetal condition was correctly predicted by the maternal urinary estriol level. Other authors have investigated specific types of complicated pregnancies and have observed that:

(1) Low estriol levels have been found in cases of unexplained premature delivery and spontaneous rupture of the membranes (14).

(2) In patients who develop toxemia during pregnancy, a significant number have low estriol levels and in general, the more severe the toxemia, the greater the reduction in estriol output. In thirty-two cases of toxemia, Greene and Touchstone (15) found that in nine of thirteen births preceeded by estriol output of 4.0 mg./day

or less, fetal death occurred. In the remaining four cases, prompt Ceasarean section yielded living babies.

(3) Low urinary estriol levels have been found in cases of pyelitis. With the advent of high body temperature and pyuria, the patient's uterus may become hyperactive and premature labor may result. In six such cases, Taylor et al (14) found the urinary estriol levels to range from 2.0 - 5.0 mg./day compared to a normal range by their method of 6.0 - 15 mg./day. Although the mechanism of this reduction is not yet understood, Taylor suggests that bacterial or enzyme activity in the urine may destroy estriol. Also, low urinary estriol levels could represent decreased production, decreased excretion, or decreased intermediary metabolism of the estrogens.

(4) Measurements of urinary estriol excretion in Rh sensitized cases offer little diagnostic aid. Scommegna and Chattoraj (16) studied fifteen cases of erythroblastosis fetalis and found normal, sometimes elevated, levels of estriol in the maternal urine. Only when perinatal death had occurred did estriol excretion drop to very low levels. Taylor et al (17) in a study of nine cases of erythroblastosis fetalis found elevated

levels of urinary estriol and suggested that increased placental mass or function may be related to the increased estriol production.

(5) In pregnancies complicated by diabetes, estriol determinations are of little value. Taylor et al (18) carried out serial studies of estriol excreted by seven pregnant, diabetic patients and found that during the last half of pregnancy, estriol excretion by the seven affected patients did not differ significantly from non-diabetic patients with normal pregnancies.

Over three hundred urinary estriol level determinations have been performed in the Department of Clinical Laboratories, University of Alberta Hospital. A study of the clinical records suggests that estriol values below 10.0 mg./day, or rapidly falling levels during a serial study, suggest fetal distress and values below 4.0 mg./day indicate almost certain fetal death.

During pregnancy, the rise in progesterone production, mainly pregnanediol, is striking. Klopper and Billewicz (12) studied fifty-six normal pregnancies at regular intervals from the sixth week of gestation to the forty-first week. They found that pregnanediol excretion rose from 6.0 mg./day to 50 mg./day. Most investigators have observed a leveling off of urinary pregnanediol excretion after thirty-six weeks gestation, due to the placenta being fully grown at

that time (11).

One of the most frequently used clinical applications of urinary pregnanediol determinations lies in cases of threatened abortion. Shearman (19) studied five cases which resulted in abortion during the first trimester of pregnancy. In all cases, serial urinary pregnanediol determinations showed falling levels of pregnanediol. At the time of abortion, levels ranged from 2.0 - 4.0 mg./day, compared to Shearman's normal range of 5.0 - 15 mg./day in the first trimester of pregnancy.

Low pregnanediol levels have been found in most toxemias of pregnancy (19-21). It is probable that this reflects decreased placental function, however, alterations in progesterone metabolism or renal clearance of pregnanediol could contribute to low urinary pregnanediol levels.

Russel et al (21) have found that urinary pregnanediol levels in diabetics are within the normal pregnancy range. Also, in Rh sensitized cases, urinary pregnanediol levels offer no insight into the well-being of the fetus (21).

Recently, urinary pregnanolone measurements have been suggested to specifically reflect placental function (8). Larsen and Engstrom reported that after thirty-five weeks gestation, less than 4.0 mg./day of urinary pregnanolone indicated placental dysfunction. Furthur studies of pregnanolone in relation to placental function have yet to be reported.

Thus, it may be of value to determine urinary estriol, pregnanediol and even pregnanolone levels in suspected complications of pregnancy. In various abnormal pregnancies the excretion values obtained could give a more complete picture of the fetus and the placenta, both separately and as a functioning unit.

Urinary pregnanetriol is generally accepted to be of adrenal origin only, yet Harkness and Love (22) have shown increased urinary pregnanetriol levels in the last trimester of pregnancy. They have also demonstrated an increase in pregnanetriol excretion by an adrenalectomized, pregnant patient, thus suggesting that the feto-placental unit is probably an additional source of pregnanetriol. Determination of pregnanetriol levels during pregnancy is of little value, since the increase in pregnanetriol level throughout pregnancy is small, rising from 1.04 ± 0.49 mg./day at twenty-three weeks gestation to 2.36 ± 1.01 mg./day at thirty-seven weeks gestation (22). However, one possible application of urinary pregnanetriol measurements during pregnancy is in cases of hydatiform mole, where values have been reported up to 40 mg./day (23).

3. In various disease states not related to pregnancy

Decreased urinary estriol excretion is a consistent finding in cases of amenorrhea. In a study of twenty-four women suffering from amenorrhea, Brown et al (24)

demonstrated that an inactive endometrium is usually associated with an excretion of less than 5 mcg. of estriol/day and stimulation of the endometrium is found at levels higher than this. In cases of amenorrhea due to congenital absence of the uterus and vagina, estriol output was normal and thus normal ovarian function is possible in this type of amenorrhea.

Estriol excretion levels of less than 10 mcg./day, with little variation throughout the cycle, have been reported in cases of anovulatory menstrual cycles (24).

Cystic glandular hyperplasia is found in association with relatively constant estriol levels of 15 mcg. of estriol/ day or more. Bleeding from this type of endometrium may occur while the output is constant, rising or falling (2).

Increased urinary estriol levels are usually found associated with most tumors of the ovaries. Although few tumors invoke basic endocrine disturbances, estriol output increases due to a general destruction of the ovarian tissues (6,25,26).

The adrenals produce only a small fraction of the total body estriol, yet they possess a large potential for increasing estriol output (2). In some cases of adrenocortical tumors and adrenocortical hyperplasia, estriol output is greatly increased (27,28). Values may increase by sixty fold in severe cases of adrenocortical hyperplasia and by twenty fold in various adrenocortical tumors.

The determination of pregnanediol is useful in ascertaining whether or not ovulation has occurred. In anovulatory menstruation, the urinary pregnanediol level usually ranges from 0.8 - 1.5 mg./day. Single urinary pregnanediol determinations are of little value unless accompanied by an endometrial biopsy. However, serial determinations of urinary pregnanediol throughout the menstrual cycle give a better indication of ovulation. Rivera et al (29) have stated that in a series of artificially induced anovulatory cycles, urinary pregnanediol values exceeding 1.0 mg./day were never observed and in fact, values rarely exceeded 0.5 mg./day.

In certain adrenal diseases, urinary pregnanediol excretion can be increased, however, other metabolites such as pregnanetriol are more diagnostic than pregnanediol levels (30).

In some ovarian tumors, increased urinary pregnanediol levels have been observed, however, a definite correlation of increased pregnanediol production to the presence of ovarian tumors has not yet been established (30).

The importance of pregnanetriol excretion in adrenocortical dysfunction was first described by Kepler and Mason (31) who studied three cases of congenital adrenal hyperplasia and found urinary pregnanetriol excretion to be increased, the levels being over 5.0 mg./day. This adrenal disease is due to various types of enzyme deficiencies which block the conversion of pregnenolone

into hydrocortisone. The result is an overproduction of ACTH by the pituitary gland and thus an increase in adrenal metabolic activity (32). More recent studies have confirmed the findings of Kepler and Mason in similar cases of congenital adrenal hyperplasia (29,33). Although there are different types of this congenital abnormality, which in turn depend on the particular enzyme deficiency, the primary clinical problem is the same, i.e., the inability to produce hydrocortisone.

Additional tests are usually employed to confirm a diagnosis of adrenal hyperplasia. The administration of dexamethasone suppresses pituitary ACTH production by blocking the release of corticotropic releasing factor (CRF) from the hypothalamus. The result in normal individuals is a reduction of androgenic hormones and pregnanetriol production. In adrenal tumors, this test has little effect on the production of these hormones as the increased output is mainly controlled by the affected tissue site in the adrenal cortex. In adrenal hyperplasia (Cushing's type) increased doses of dexamethasone are required to lower the ACTH production. In congenital adrenal hyperplasia, androgenic hormones and pregnanetriol production decreases.

Since ACTH normally controls the production of adrenocortical hormones, it provides another means of pinpointing adrenal dysfunction. When ACTH is administered,

to normal individuals, the result is an increase in adrenocortical hormone production. Cawley et al (34) studied the urinary pregnanetriol output before and after ACTH stimulation and found a two to five fold increased in pregnanetriol excretion. In adrenal hyperplasia, ACTH will stimulate a much greater production of androgenic and pregnanetriol metabolites than in normal individuals (33). Lack of response to ACTH stimulation usually indicates an adrenal tumor where the surrounding unaffected parts of the gland have become completely atrophied.

From the preceding discussion, it is apparent that an investigation of the following would be of interest:

(1) Development of an analytic method for the simultaneous determination of estriol, pregnanediol and pregnanolone in pregnancy urine. This method may be particularly useful for elucidating the cause of fetal distress in some complications of pregnancy which may be due to placental insufficiency or the fetus itself.

(2) Development of a reproducible, analytic method for the simultaneous determination of pregnanediol and pregnanetriol in non-pregnancy urine. From this information, the obstetrician may be able to assess some aspects of both ovarian and adrenal function together.

(3) Development of a shorter and simpler procedure than

presently available for the measurement of estriol in non-pregnancy urine. Ready availability of estriol levels should give additional insight into ovulation and various menstrual cycle disorders.

II. EXPERIMENTAL

A. HYDROLYSIS OF STEROID CONJUGATES

Since steroids are excreted mainly as water soluble conjugates, hydrolysis of the conjugates to the free steroid form is a necessary preliminary to their extraction into an organic solvent for purposes of separation and purification.

Two basic methods are used for the hydrolysis of steroid conjugates: (1) Heating with mineral acids, and (2) treatment with the enzymes β-glucuronidase and sulfatase.

Brown and Blair (35) examined the conditions of acid hydrolysis and concluded that adding 15 - 20 volumes% of concentrated hydrochloric acid to urine and refluxing for one to two hours gave maximum recovery of steroids with minimal destruction of the liberated free steroids.

Wakabayashi and Fishman (36) studied the enzymatic hydrolysis of steroid conjugates and demonstrated that β-glucuronidase prepared from beef liver, Patella vulgata and Helix pomatia effect maximum hydrolysis at pH 4.0 to 4.5 when incubated at 37°C. for twenty four hours. Sulfatase has an optimum hydrolytic effect at pH 5.8 and

37°C. (37). Since a mixture of these enzymes is necessary for complete hydrolysis of steroid conjugates, it is impossible to satisfy both optimal conditions. Most investigators have compromised the conditions by carrying out enzymatic hydrolysis at pH 5.2 and at 37 - 39° C. for eighteen to twenty-four hours (8,28,38). Varying concentrations of the enzymes have been used with little effect on the hydrolytic efficiency, providing that an excess of the enzymes is always present during hydrolysis.

1. Acid hydrolysis vs. enzyme hydrolysis

From the literature it is obvious that acid hydrolysis is more rapid than enzyme hydrolysis, however, at the same time possesses several drawbacks. Thus, it was decided that part of this project for the development of analytic methods would necessitate a study of the two hydrolytic procedures.

The conditions for hydrolysis were as follows:

(1) Enzyme hydrolysis - Fifty ml. of urine were brought to pH 5.2 by the addition of glacial acetic acid or 10 N sodium hydroxide. To this, 10 ml. of 0.2 M sodium acetate buffer (pH 5.2) and 0.5 ml. of Glusulase (contains 100,000 Units of β-glucuronidase and 50,000 Units of sulfatase/ml.) were added. The mixture was incubated at 37°C. in a shaking water bath for 24 hours.

(2) Acid hydrolysis - To 50 ml. of urine, 7.5 ml. of

concentrated hydrochloric acid was added and the mixture refluxed for ½ hour.

The analysis of ten different pregnancy urine specimens was carried out in order to decide which method of hydrolysis would give maximum recovery of estriol from its conjugates. One of 2 duplicate aliquots from a pregnancy urine specimen was subjected to enzyme hydrolysis while the other was hydrolyzed with acid. After hydrolysis was complete, estriol was extracted from the urine and determined by Gas-Liquid Chromatography (GLC), using the procedure to be described later. It can be seen from Table I that an average of 19% more estriol was obtained when enzyme hydrolysis was employed. This agrees well with the findings of Wotiz and Martin (39).

TABLE I

Comparison of the amounts of estriol recovered from pregnancy urine following acid or enzyme hydrolysis.

Urine Specimen	Acid Hydrolysis (mg.estriol per 24 hr.)	Enzyme Hydrolysis (mg. estriol per 24 hr.)	% Difference $\frac{(\text{enzyme} - \text{acid})}{\text{enzyme}} \times 100$
1	8.9	11.2	21
2	15.8	18.8	16
3	5.6	8.5	34
4	26.5	29.0	9
5	6.7	8.5	21
6	28.5	31.0	8
7	15.0	21.6	31
8	27.0	35.8	25
9	22.1	24.6	10
10	21.2	24.6	16
Average loss by acid hydrolysis =			19%

A more striking example of steroid destruction during acid hydrolysis was observed when pregnanediol in pregnancy urine was investigated. Compared to enzyme hydrolysis, up to 50% less pregnanediol was recovered from urine specimens when acid hydrolysis was used. Astwood and Seegar-Jones (40) have suggested a loss of pregnanediol when using acid hydrolysis, however, this problem has generally not been described in the literature. Destruction of pregnanediol by acid hydrolysis may be prevented by hydrolysis-extraction in the presence of toluene (40). However, this is not applicable when estriol measurements are also desired, since estriol is not quantitatively extracted by toluene. Since it was proposed to develop a simultaneous method for pregnanediol and estriol measurement, this method of hydrolysis could not be used.

2. Effect of urinary glucose, highly concentrated urine and the urinary antiseptic, Mandelamine, on the recovery of estriol following acid hydrolysis.

A recent study of acid hydrolysis of urinary estriol conjugates has revealed further problems following acid hydrolysis. Low estriol measurements were obtained in the following situations (41):

(1) Highly concentrated pregnancy urine (as revealed by measuring the specific gravity).

(2) Urine of diabetic patients who exhibit some degree of glycosuria.

(3) Urine from patients being treated with the urinary tract antiseptic Mandelamine.

An investigation of these problems was made by using both acid and enzyme hydrolysis on such urine specimens.

A highly concentrated urine (specific gravity 1.030) was hydrolyzed by acid and enzyme hydrolysis. Also, the urine was diluted ½ with water and hydrolyzed by both methods. The extraction and GLC of estriol was performed using the procedure to be described later. It was found that a 12% greater yield of estriol from the diluted samples resulted. Schindler et al (41) have reported up to 20% increase in estriol recovery for the same dilution factor of highly concentrated urines. Enzyme hydrolysis was not affected by the degree to which the urine specimen was concentrated. Pregnanediol recovery was not affected by either hydrolytic procedure.

In an attempt to elucidate the reason for estriol loss in highly concentrated urines, both urea and sodium chloride were added to a urine of relatively low specific gravity (1.015) to increase the specific gravity. The recovery of estriol was not diminished by the addition of either solute.

To investigate the effect of urinary glucose on the hydrolysis of estriol conjugates, the analysis of five different pregnancy urine specimens containing glucose (0.75 - 2.00 mg glucose/100 ml. of urine) was carried out

in duplicate. One of two duplicate aliquots from a urine sample was subjected to enzyme hydrolysis and the other aliquot subjected to acid hydrolysis. The results of this study, as summarized in Table II, show an average of 40% less estriol in urine specimens that were hydrolyzed by mineral acid treatment. Glucose did not affect urinary pregnanediol recovery.

At the moment, there is no explanation for the loss of estriol in highly concentrated urines or in urines containing glucose. The problem can be avoided by either diluting the urine (41) or using enzyme hydrolysis.

TABLE II

Comparison of enzyme and acid hydrolysis of estriol conjugates in pregnancy urine containing glucose.

Urine Specimen	Acid Hydrolysis (mg. estriol per 24 hr.)	Enzyme Hydrolysis (mg. estriol per 24 hr.)	% Difference
1	8.4	14.0	40
2	17.0	31.0	45
3	3.8	6.1	38
4	17.8	30.0	41
5	11.6	17.6	34
Average loss by acid hydrolysis =			40%

One pregnant patient receiving Mandelamine was investigated. As Touchstone et al (42) have shown, estriol could not be detected when using acid hydrolysis on the urine from a patient being given this drug. However, when enzyme hydrolysis was used, accurate quantitation of estriol was possible. Figure III shows a GL chromatogram of the patient's urine following acid hydrolysis, while Figure IV demonstrates that estriol was hydrolyzed and extracted efficiently in the presence of Mandelamine when enzyme hydrolysis was employed. Quantitation of pregnanediol was not affected by either hydrolytic procedure, other than the usual effect of acid hydrolysis previously described. Touchstone et al (42) suggest that acid hydrolysis releases formaldehyde from hexamethylenetetramine (a constituent of the drug) and that formaldehyde complexes with estriol, preventing its extraction into the organic solvent.

Acid hydrolysis has been criticized for producing many degradation products of steroids and other constituents of urine (43). The reason for the widespread use of this procedure probably lies in its simplicity, low cost and speed.

Enzyme hydrolysis also has several short-comings. Stempfel et al (44) have shown inhibition of enzyme hydrolysis by moderately large concentrations of salicylates, heparin and ascorbic acid in the urine.

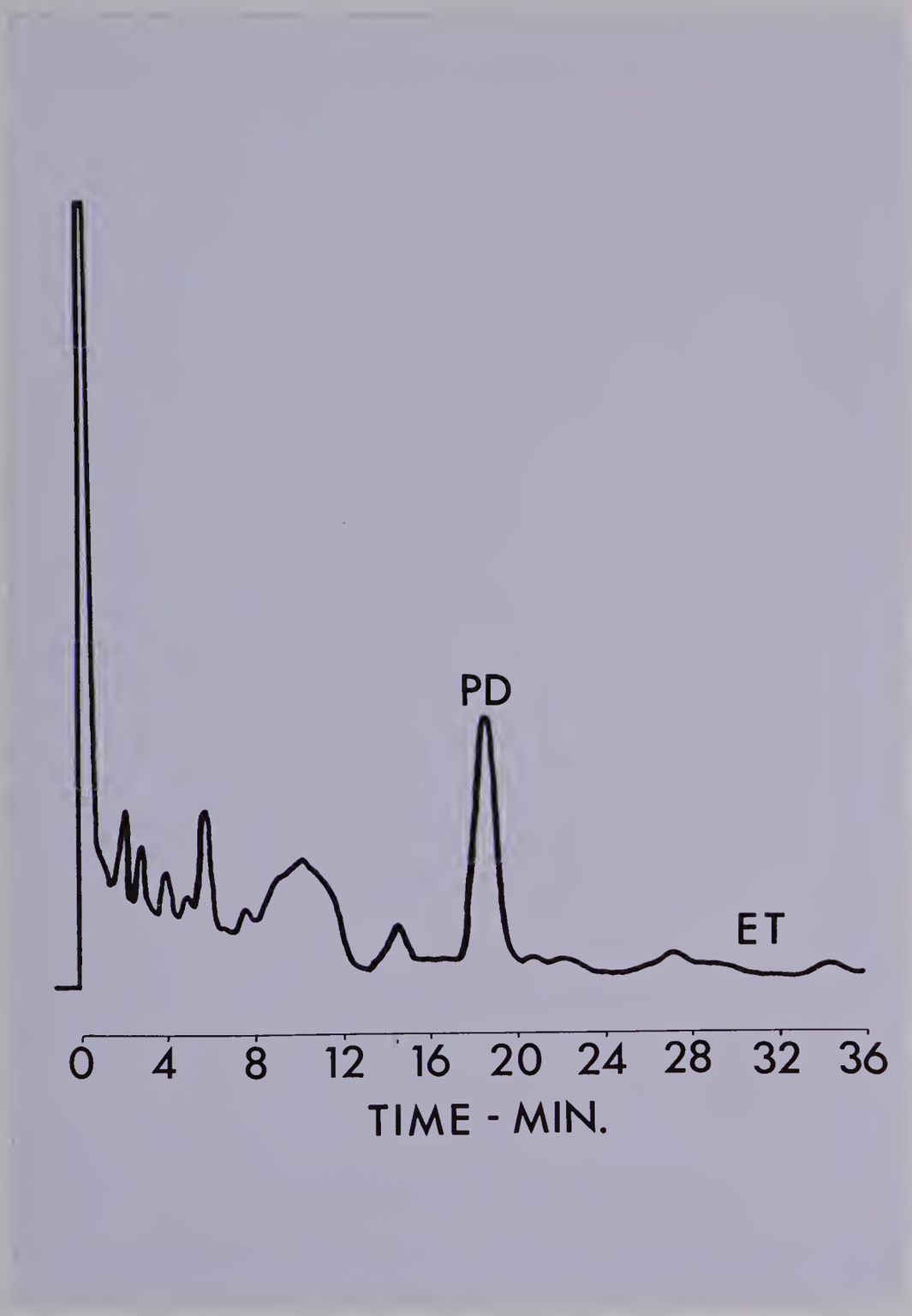

FIGURE III. GL chromatogram showing the effect of Mandelamine on estriol recovery from pregnancy urine when using acid hydrolysis. The compounds are the TMSi ether derivatives of estriol (ET) and pregnanediol (PD).

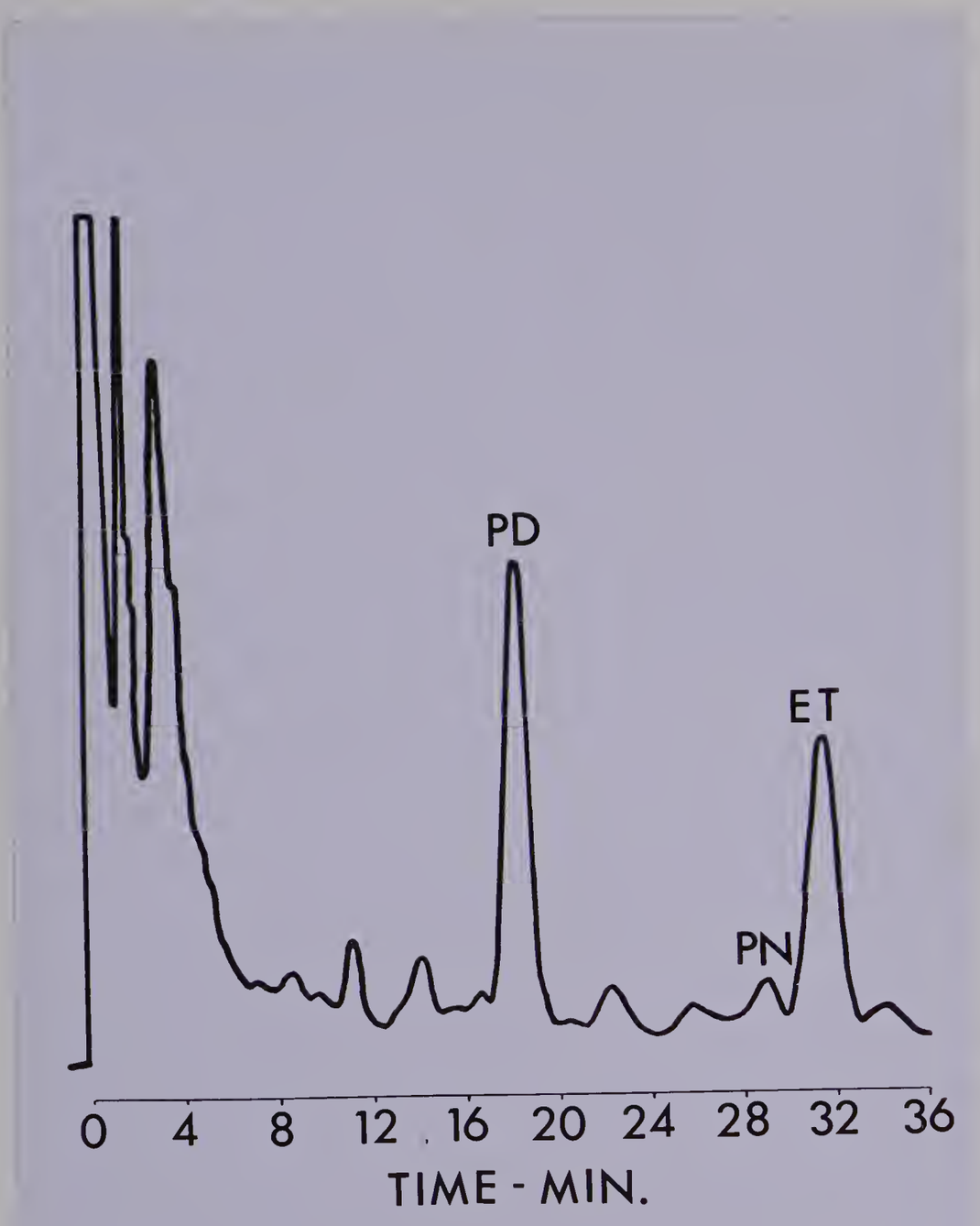

FIGURE IV. GL chromatogram of estriol and pregnanediol extracted from an enzymatically hydrolyzed pregnancy urine containing Mandelamine. The compounds are the TMSi ether derivatives of estriol (ET)

B. EXTRACTION PROCEDURE

The method of extracting and isolating urinary steroids from other constituents in the urine is basically that of Larsen and Engstrom (8). When enzyme hydrolysis was used, 8.0 ml. of concentrated hydrochloric acid was added before the extractions to insure complete recovery of the desired steroids from the urine. A mechanical shaker (Virtis Extractomatic, Gardiner, N.Y.) equipped with six separatory funnels was used throughout the extraction procedure.

The steroids were extracted from the hydrolyzed urine with three 50 ml. portions of ether. When enzyme hydrolysis was used, emulsions invariably resulted after the first ether extraction. It was found that two 75 ml. ether extractions followed by a 50 ml. ether extraction minimized the formation of emulsions. If an emulsion did result after the first extraction, the two phases were allowed to separate out for about ½ hr. and the following two ether extractions were performed on the aqueous phase. The three ether phases were combined and vigorously shaken, resulting in breakdown of the emulsion.

Two 50 ml. 9% sodium bicarbonate washes of the ether phase were next performed. This removed traces of hydrochloric acid and some of the acidic chromogens. The sodium bicarbonate washes were discarded.

The ether phase was then extracted with two 50 ml. portions of 1 N sodium hydroxide. This extraction separated the estrogens from the progesterones. Since the estrogens possess acidic properties, they are extracted into sodium hydroxide, whilst the progesterones remain in the ether phase. This extraction step was used only when separate analysis of estriol and the progesterones (pregnanediol and pregnanetriol) was required, as in non-pregnancy urine specimens.

The sodium hydroxide fraction was then neutralized with concentrated hydrochloric acid (about 9.0 ml.) and then extracted three times with 50 ml. portions of ether, The ether phases were combined and traces of water were removed by the addition of a few grams of anhydrous sodium sulfate. The ether was then evaporated to dryness using a rotary evaporator *in vacuo* at 50°C. The resulting residue was transferred with small aliquots of acetone to a small volume round bottom flask and again evaporated to dryness. The flask was tightly stoppered to protect the residue from moisture.

The ether fraction originally containing the progesterones was treated in the same manner as the final ether extraction containing the estrogens, i.e., evaporation and transfer of the residue to a small volume flask.

Throughout this work, chemicals of A.C.S grade were always used. Solvents such as acetone, chloroform and

pyridine were of special purity (Fisher spectranalyzed).

C. DERIVATIZATION FOR GLC

Derivative formation of steroid prior to GLC analysis is essential. Steroids, such as estriol, possess several polar functional groups and tend to undergo thermal decomposition or irreversible adsorption onto active sites of the column to a greater extent than non-polar steroids. Thus, conversion of free steroids into relatively non-polar derivatives is desirable (45).

Also, adequate separations are frequently not attained between free steroids. The formation of derivatives produces greater molecular weight differences between similar steroids, such as the estrogens, allowing better separation (46). A comparison of Figure V and Figure VI illustrates the lack of separation of estrone, estradiol and estriol when GLC analysis is performed on the free steroid form of the three estrogens. Also, lack of symmetry in the estriol peak in Figure V suggests column adsorption of free estriol. It can be seen that upon derivatization of estrone, estradiol and estriol, these problems of separation and column adsorption are readily overcome.

However, only certain types of derivatives are suitable for GLC analysis. A perferred derivative should be thermostable, possess an appropriate degree of volatility

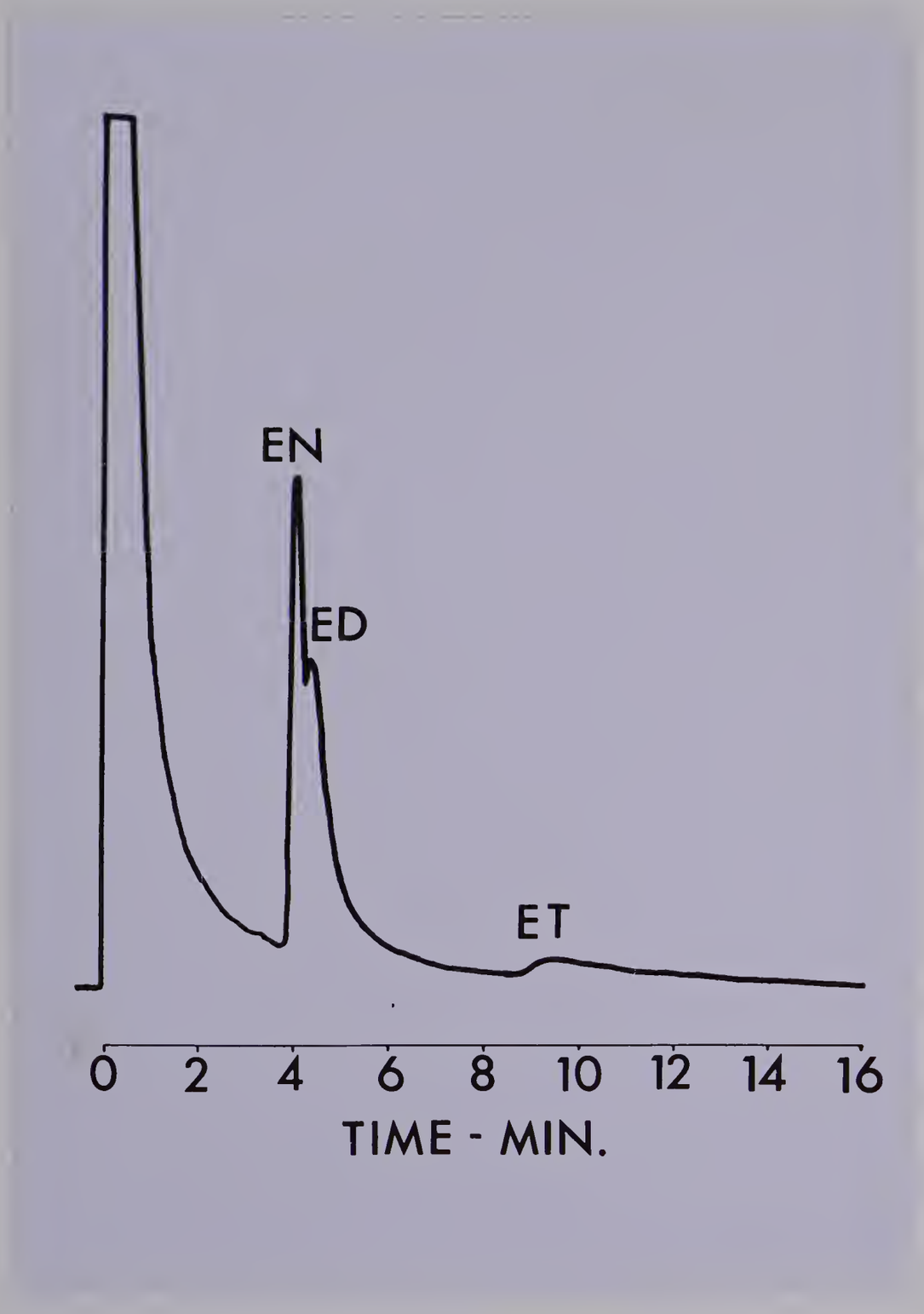

FIGURE V. The GLC analysis of a mixture of estrone (EN), estradiol (ED) and estriol (ET) in the free steroid form.

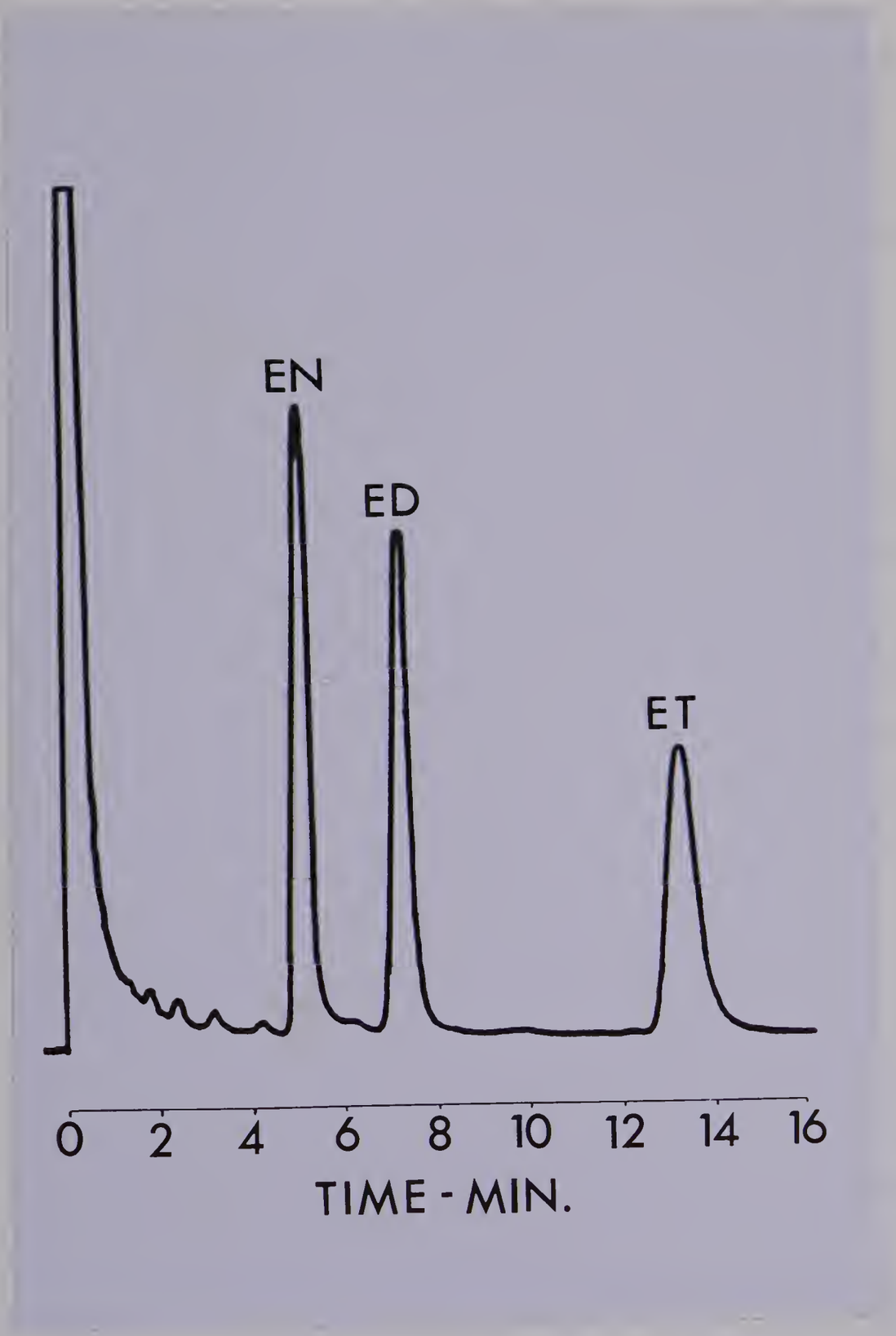

FIGURE VI. The GLC analysis of a mixture of estrone (EN), estradiol (ED) and estriol (ET) as acetate derivatives.

and have polar properties which are considerably less than the parent steroid (45). Types of derivatives presently used in steroid GLC analysis include trimethylsilyl ethers, acetic acid esters, trifluoracetyl esters and methyl ethers.

1. Acetate derivatives

The use of acetate derivatives of steroids for GLC analysis, particularly the estrogens, has been investigated and highly recommended by Wotiz and Martin (47). Wotiz (46) has shown that a 5:1 mixture of acetic anhydride and pyridine gives optimal conversion of the free estrogens to their acetate derivatives. The reaction is carried out in a glass-stoppered flask at 65°C. for ½ hour. Progesterones react somewhat slower and require at least one hr. at 65°C. or standing at room temperature overnite (8).

A study of the reaction times of the estrogens (estrone, estradiol and estriol) and progesterones (pregnanolone, pregnanediol and pregnanetriol) was made. The acetate derivatives were formed as described above. As expected, the reaction time increased as the number of reactive hydroxyl groups on the steroid nucleus increased. Acetylation of estriol was complete in thirty minutes. The progesterones had completely reacted in thirty min. when using a standard mixture, whereas sixty min. were required for complete acetylation of pregnanetriol from a urine extract. Competitive acetylation reactions with

other urinary constituents probably accounted for this finding.

The stability of the estrogen acetates under GLC conditions is excellent. However, a problem encountered during GLC analysis lies in the fact that an appreciable amount of adsorption of these compounds onto the column occurs. This problem has been partially eliminated by "priming" the column prior to GLC analysis. This involves making several successive injections of steroid acetate derivatives onto the column. The active adsorption sites are blocked by this "priming" process, but the procedure takes some time.

2. Trimethylsilyl ether derivatives

The application of trimethylsilyl (TMSi) ether derivatives to GLC analysis of steroids was first investigated by Luukkainen et al (48). Separation of the estrogens and some of the progesterones was found to be excellent. A major advantage of this type of derivative was found when analysis of the steroids was performed on selective stationary phase columns, such as Neopentyl Gycol Succinate (NGS). With these derivatives, no selective group retention effects were observed. This did not occur when the acetate derivatives were employed, as several hours were required for their elution (49). Also, adsorption of the TMSi ether derivatives onto the column during GLC was absent.

The method of Makita and Wells (50) for preparing TMSi ether derivatives was one of the first published and is still widely used. They showed that using a 10:3 mixture of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in pyridine resulted in complete derivatization of fecal sterols within 10 minutes. However, most procedures which use TMSi ether derivatives for urinary steroid GLC analysis require that HMDS and TMCS react with the steroid mixture overnite at room temperature.

Recently, Horning et at (51) showed that Bis-(trimethylsilyl)-acetamide (BSA) was a very effective silylating reagent and when TMCS was added, the reaction was rapid.

The formation of TMSi ether derivatives of the estrogens (estrone, estradiol and estriol) and the progesterones (pregnanolone, pregnanediol and pregnanetriol) was investigated. The derivatives were formed by adding 1 ml. of pyridine, 0.5 ml. of BSA and 25 µl. of TMCS to a urine extract containing the steroids. It was found that the reaction was complete after fifteen min. when carried out at room temperature. As in the formation of acetate derivatives, it was observed that a standard mixture of the steroids reacted faster than steroids from a urine extract.

The BSA-TMCS method has definite advantages over the HMDS-TMCS method of forming TMSi ether derivatives. When HMDS and TMCS are mixed, a white precipitate forms.

Langer et al (52) have demonstrated by infrared analysis that the precipitate is ammonium chloride, which resulted from the overall reaction:

$$3ROH + (CH_3)_3SiCl + (CH_3)_3SiNHSi(CH_3)_3 \longrightarrow 3RO\text{-}Si(CH_3)_3 + NH_4Cl$$

where R represents the steroid nucleus.

Although the presence of ammonium chloride does not affect the TMSi ether derivatives, it presents a problem when the mixture is injected onto the column for GLC analysis. This can be circumvented by centrifuging the mixture and removing the clear supernatant for GLC analysis. However, this adds a furthur step to the method.

The reaction time for the HMDS-TMCS procedure is also a disadvantage, taking overnite at room temperature for complete formation of the derivatives. Although the reaction is complete in thirty min. when incubated at 56° C. (34), the possibility of introducing moisture to the mixture and the volatility of the solvents at that temperature discourages use of this method.

The main disadvantage common to both methods of preparing TMSi ether derivatives is that the ether linkage of the derivatives is readily cleaved in the presence of moisture. Thus, the preparation and handling of TMSi ether derivatives under anhydrous conditions is essential.

D. GAS-LIQUID CHROMATOGRAPHY

Gas-liquid chromatography is a form of partition chromatography in which the mobile phase is an inert gas and the liquid phase is a liquid film held in place on a solid, inert support. Compounds which are volatile at the column temperature used, distribute themselves between the two phases and the order of their elution from the column is determined by their respective partition coefficients.

As the compounds are eluted from the column, they enter a detector cell and the detector responses are registered in the electrometer component. These responses are amplified accordingly and recorded as distinct peaks on the chromatogram.

Quantitation of peaks is accomplished by measuring the area under the curve. Since a typical peak represents an isosceles triangle, the area can be calculated by multiplying the half-width of the peak by the height of the peak. The area is usually expressed in mm^2.

1. Instrumentation

A Hewlett-Packard (F&M Scientific) Model 402 dual column gas chromatograph equipped with a hydrogen flame ionization detector and a temperature programmer was used.

A six foot glass U-column with an internal diameter of 3 mm. was packed with the stationary phase, a

commercially prepared packing.

Helium (the mobile phase) was used as the carrier gas. Since a flame ionization detector was used, a mixture of hydrogen and air entering the detector cell was necessary. Throughout the experimental work, the flow rates of hydrogen, helium and air were maintained at 30, 60 and 240 ml./min. (1:2:8) respectively, as recommended in the instrument's instruction manual.

Thermal conductivity detection systems have been used for steroid work, however, the level of sensitivity is usually not satisfactory (45). Sensitivity is greatly enhanced when an ionization detector is employed.

The hydrogen flame ionization detector depends on the electrical conduction properties of the carrier gas and organic compounds contained within it. As a compound is swept into the hydrogen flame by the carrier gas, a certain percentage of the molecules are ionized. The extent of this ionization depends upon the nature of the compound and the flame temperature. By using a battery source, a potential is impressed between the collector (usually the anode) and the jet (usually the cathode). As the electrons are attracted to the collector, a voltage drop occurs. This change in voltage is registered in the electrometer, amplified and the output presented on a potentiometric recorder. The degree to which the voltage is amplified by the electrometer is controlled by "range"

and "attenuation" settings.

2. Operational conditions

Many variables in GLC must be ideally adjusted for adequate separation, detection and quantitation of the desired compounds. These include the type and amount of liquid phase, temperature (isothermal or programmed) and the gas flow rates.

Two liquid phases, both widely used in steroid GLC, were investigated.

OV-1 (a methylsiloxane polymer) is a non-selective liquid phase. Non-selective liquid phases show little selective retention effects for steroids with different functional groups. Thus, separation of various steroids is mainly achieved by differences in molecular size, shape and volatility. OV-1 has the separation characteristics of the popular liquid phase SE-30 (also a methysiloxane polymer), but has greater temperature stability (51). Figure VII shows the separation characteristics of a standard mixture of estrogen and progesterone derivatives on 3% OV-1 (w/w)liquid phase.

NGS is a selective liquid phase. A selective liquid phase separates steroids on the basis of their differing functional groups. The selective properties of NGS enables separation of epimers and isomers of a steroid and also shows selective retention effects for carbon-carbon unsaturation (49). The separation of various steroids thus depends on their functional groups, however, molecular

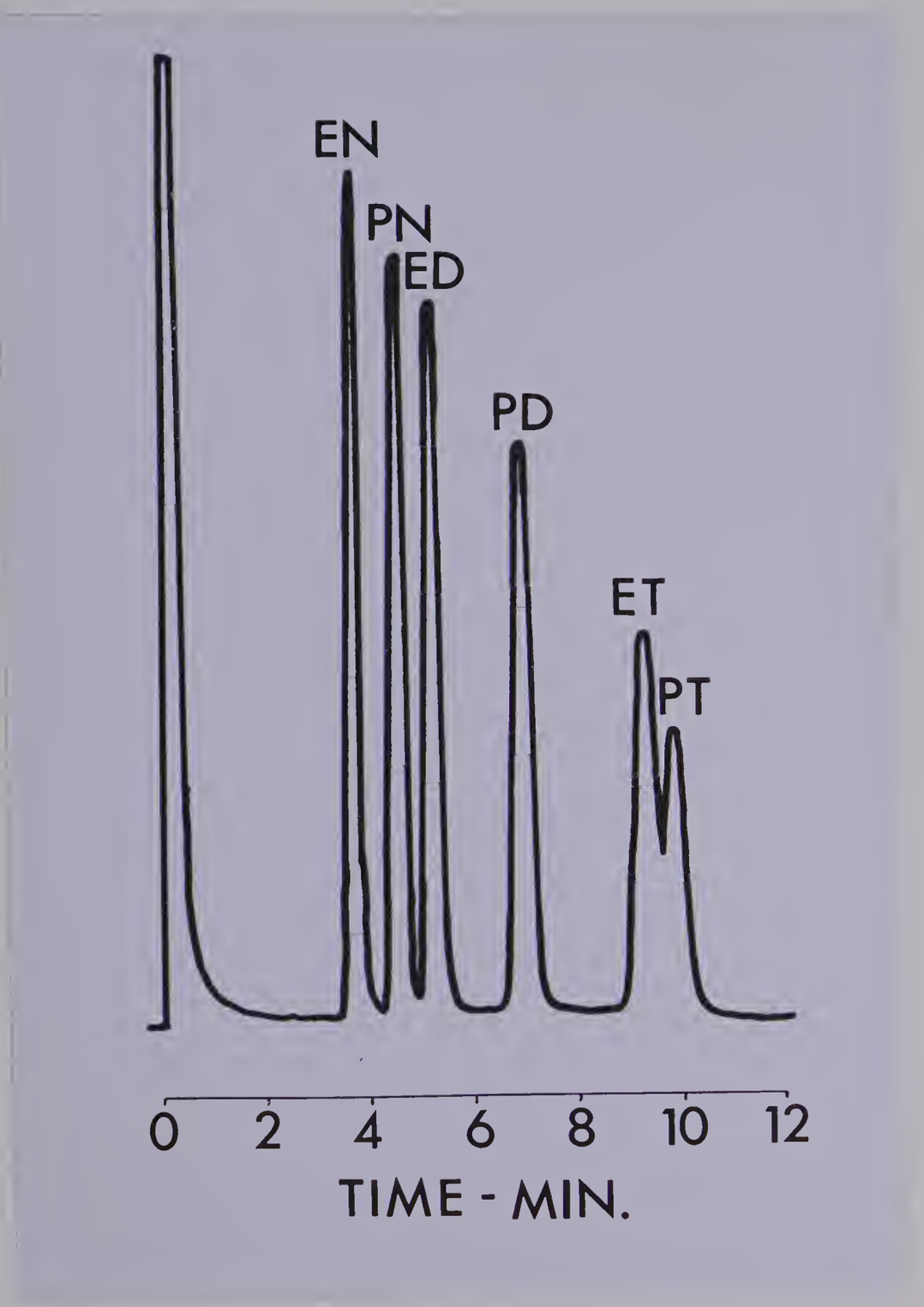

FIGURE VII. The GLC analysis of a mixture of estrogen and progesterone standards, using the liquid phase coating OV-1. The compounds are the TMSi ether derivatives of estrone (EN), estradiol (ED), estriol (ET), pregnanolone (PN), pregnanediol (PD) and pregnanetriol (PT).

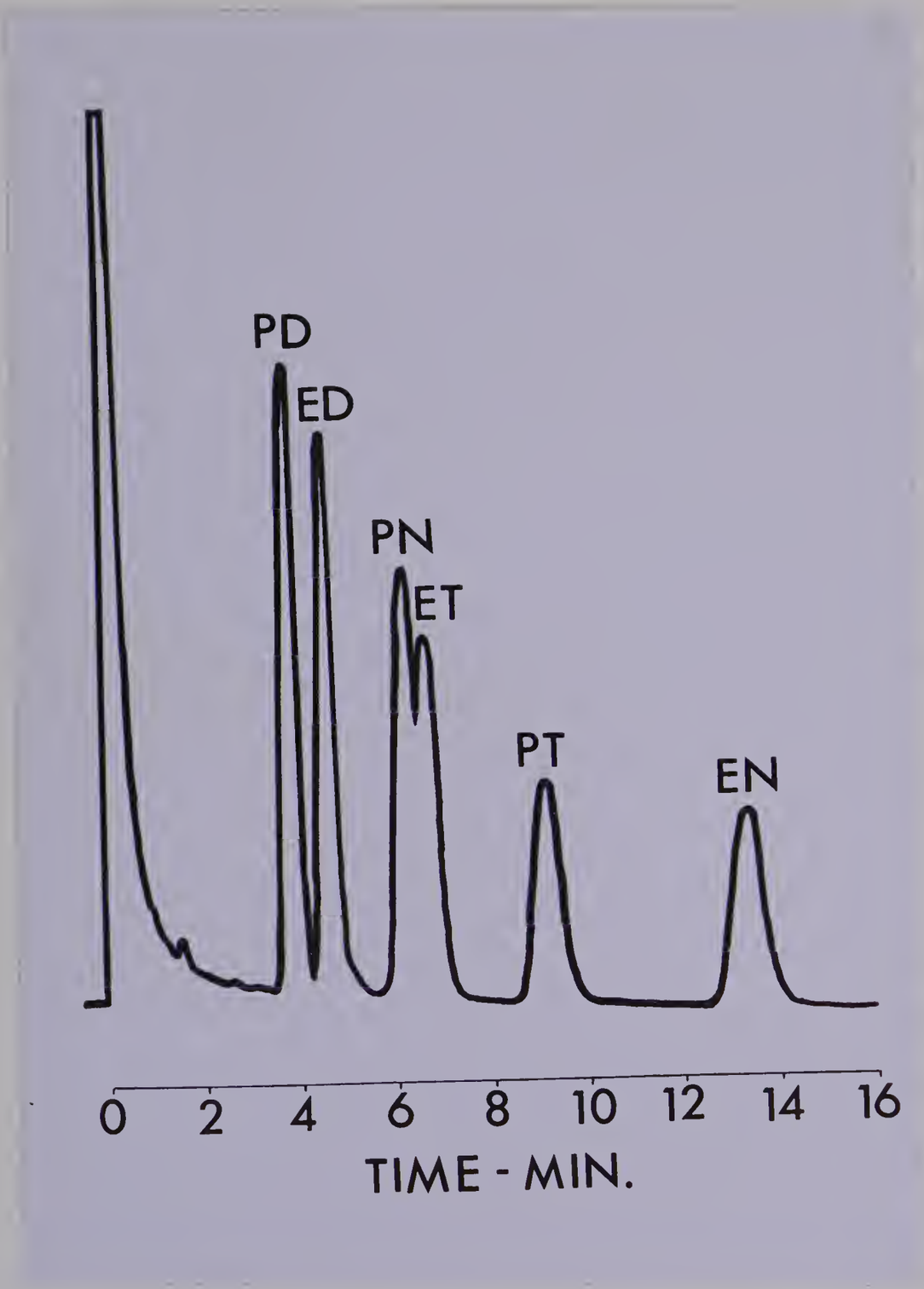

FIGURE VIII. The GLC analysis of a mixture of estrogen and progesterone standards, using the liquid phase coating NGS. The compounds are the TMSi ether derivatives of estrone (EN), estradiol (ED), estriol (ET), pregnanolone (PN), pregnanediol (PD) and pregnanetriol (PT).

size and shape also determines their separation to some extent. Figure VIII shows the separation characteristics of a mixture of estrogen and progesterone standards, as TMSi ether derivatives, on 2% NGS (w/w). Comparison of Figure VIII and Figure VII shows that neither liquid phase presented any significant advantage over the other when a mixture of estrogen and progesterone standards was analyzed.

However, when a urine extract was analyzed, the OV-1 liquid phase was unable to satisfactorily separate pregnanediol and pregnanolone from some unknown compounds which possessed similar retention characteristics. Although identification of these interfering compounds was not done, Barry et al (53) have suggested that they are isomers of pregnanediol and pregnanolone. Most of the time these isomers did not appear to significantly affect quantitation of pregnanediol and pregnanolone, but occasions did arise in which the interfering peaks prevented accurate measurement of the two progesterones. Comparison of Figure IX and Figure X shows the inability of the liquid phase coating OV-1 to separate pregnanediol and pregnanolone from their isomers. In contrast, the liquid phase coating NGS readily separated pregnanediol and pregnanolone from their isomers and permitted accurate quantitation of the two progesterones.

The amount of liquid phase coating on the column

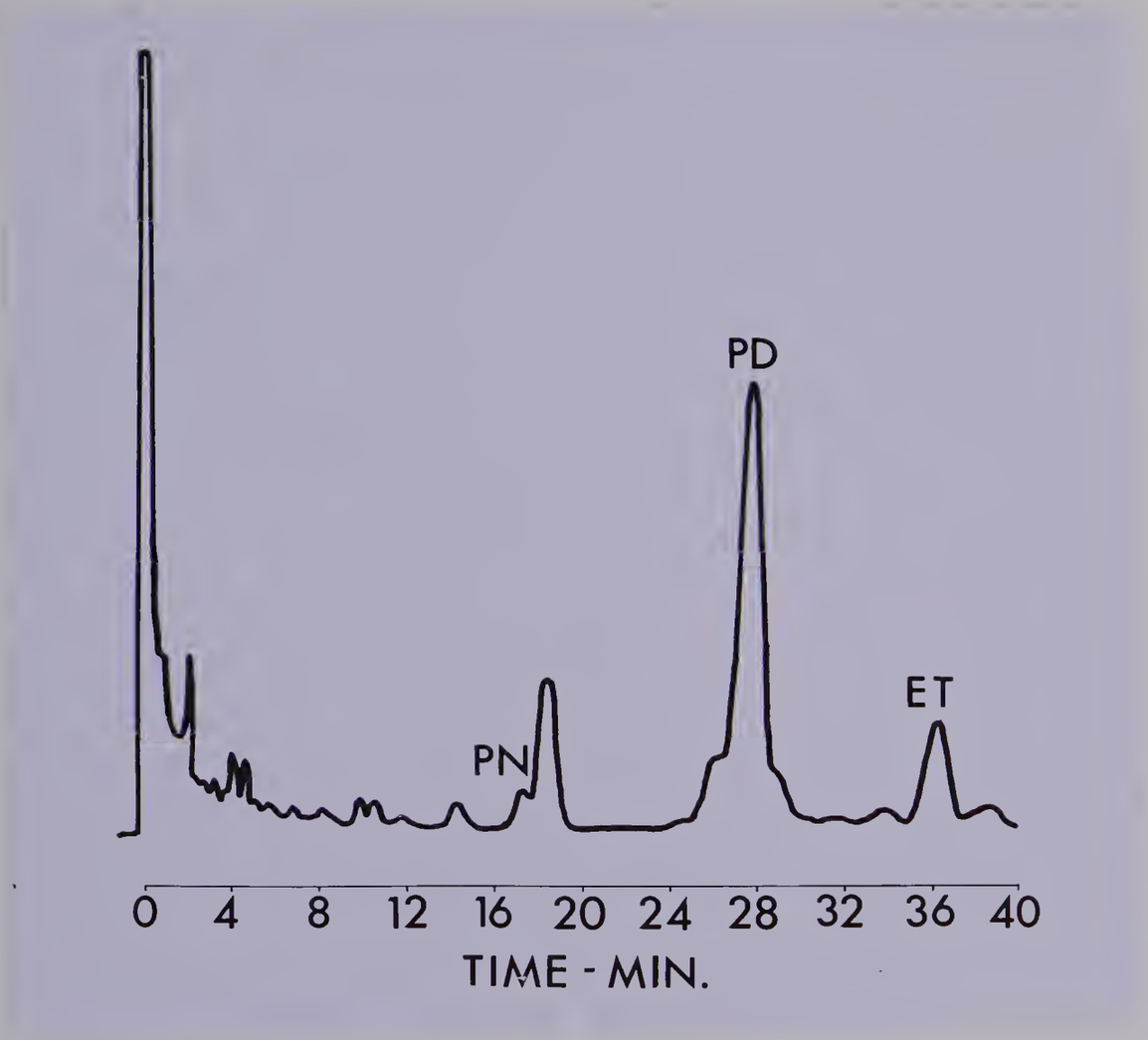

FIGURE IX. The GLC analysis of urinary estriol, pregnanediol and pregnanolone, using 3% OV-1 liquid phase coating. The compounds are the TMSi ether derivatives of estriol (ET), pregnanediol (PD) and pregnanolone (PN).

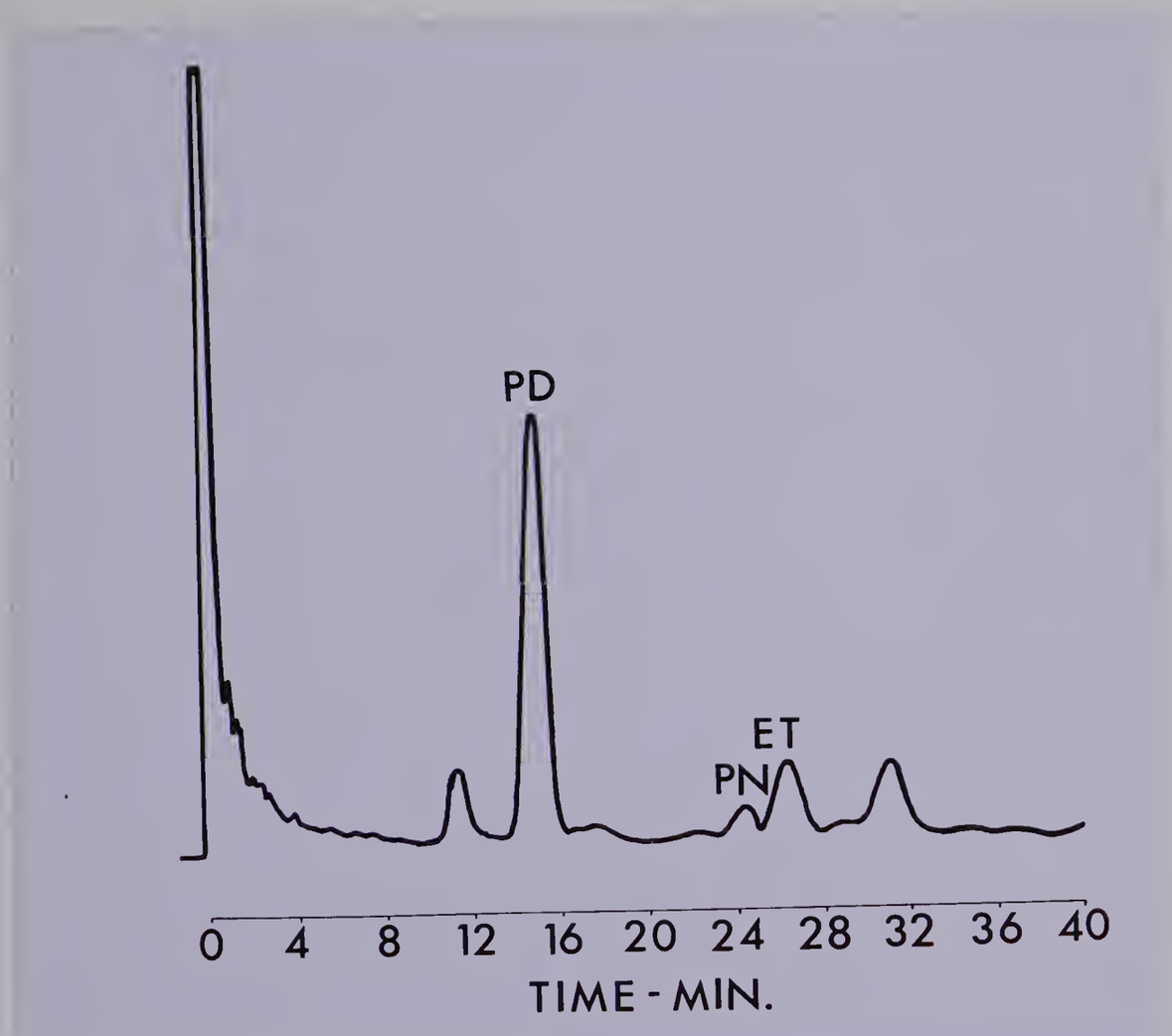

FIGURE X. The GLC analysis of urinary estriol, pregnanediol and pregnanolone, using 2% NGS liquid phase coating. The compounds are the TMSi ether derivatives of estriol (ET), pregnanediol (PD) and pregnanolone (PN).

packing is important. If too little is present, the support will not be adequately coated and the separation characteristics of the column will be affected by the adsorption characteristics of the support. If too much liquid phase is present, excessive column "bleeding" and "tailing" of peaks occurs. Liquid phase concentrations below 1% and above 4% have rarely been successfully used in steroid GLC. During this experimental work, 2% NGS and 3% OV-1 were routinely used.

The liquid phase support is usually an inert substance such as diatomaceous earth. The type of support has a limited effect on the separation characteristics of the column as a whole. A smaller particle size results in a narrower peak width, but the degree to which this may be advantageously used is limited by the need for higher pressures to force the carrier gas through the column. Treatment of the support with acid and alkali washes before coating it with a liquid phase usually prevents any contaminants from the support material affecting the separation characteristics of the column.

The effect of temperature on GLC presents a useful means for manipulating the retention characteristics of various compounds. Three temperature controls are necessary for GLC, but only the column temperature affects the retention ability of the column. The flash heater is necessary for instantaneous vaporization of the sample

and is usually set about 40°C. above the column temperature. The detector temperature, usually set about 20°C. above the column temperature, provides a hot atmosphere for ionization and detection of compounds. It also prevents condensation of components from the injected mixture within the detector cell.

Gas-Liquid Chromatography of various steroids is usually carried out at temperatures between 200° and 260°C. Temperatures beyond these limits usually affect the retention times of the steroids such that their measurement is unreliable or their separation is inadequate. Most GLC procedures for steroid analysis use isothermal conditions. However, when several different steroids are to be determined simultaneously or very small amounts of some steroids in a complex mixture are subjected to isothermal conditions, inadequate separation from unknown urinary constituents usually results. To overcome some of these problems, programmed temperature GLC has been used (54,55).

Since column temperature has a greater effect on the GLC process than any other operational variable, it can be manipulated to give better separation of components in a mixture. At a low temperature, elution of components with low partition coefficients progresses normally but components of the mixture with a high partition coefficient will remain near the head of the column. As the column

temperature is raised (usually in a linear fashion), the partition coefficients of these retained components changes, favoring the mobile (gas) phase (56). Thus, increased separation of the components from each other is achieved by virtue of their partition coefficient differences.

A disadvantage of temperature programming lies in the time required for elution of most components of a mixture. Instead of a compound taking a few minutes to be eluted, it may take several times this amount when programmed temperature GLC is employed. The length of time, of course, depends on the initial temperature of the column and the rate of programming. Also, as the retention time increases, the peak width broadens with a corresponding decrease in peak height. Thus, higher sensitivity settings of the electrometer are required.

Several types of temperature programmes were investigated with respect to the separation of a mixture of estrogen and progesterone derivatives. It was found that after sample injection, an initial ten minute delay at 210°C. followed by linear temperature programming at the rate of ½°C./min. gave the desired separation of the estrogen and progesterone mixture. Although a lower initial temperature would have resulted in still better separation, it was felt that the additional time required for elution of the steroids did not justify the improvement

in separation. Figures VII and XI show the differences between separation characteristics of various steroids when temperature programming is employed instead of isothermal conditions.

Fine control of the gas flow rates, especially helium, is essential for reproducible results. A constant pressure of each gas is maintained by use of a regulator valve on the gas supply tank and a fine needle valve control within the instrument further regulates the flow rate. By calibrating the needle valve gauge with a flowmeter, accurate and constant flow rates are obtainable. When using programmed temperature GLC, the change in helium flow rate due to temperature change is usually compensated by a form of differential flow controller, as supplied in the Hewlett-Packard 402 Gas Chromatograph.

By increasing the helium flow rate, the retention times of various compounds decrease. This can be used with advantage up to the point where the hydrogen flame is blown out after sample injection. This is not only due to the increased helium flow rate, but to the combustion of the injected solvent, which forms a sudden burst of pressure through the column.

Although the air and hydrogen flow rate control need not be as precise as the helium flow rate, a reasonably accurate mixture of the two in the detector cell is desirable. Changing the flow rates and/or ratios of these two gases

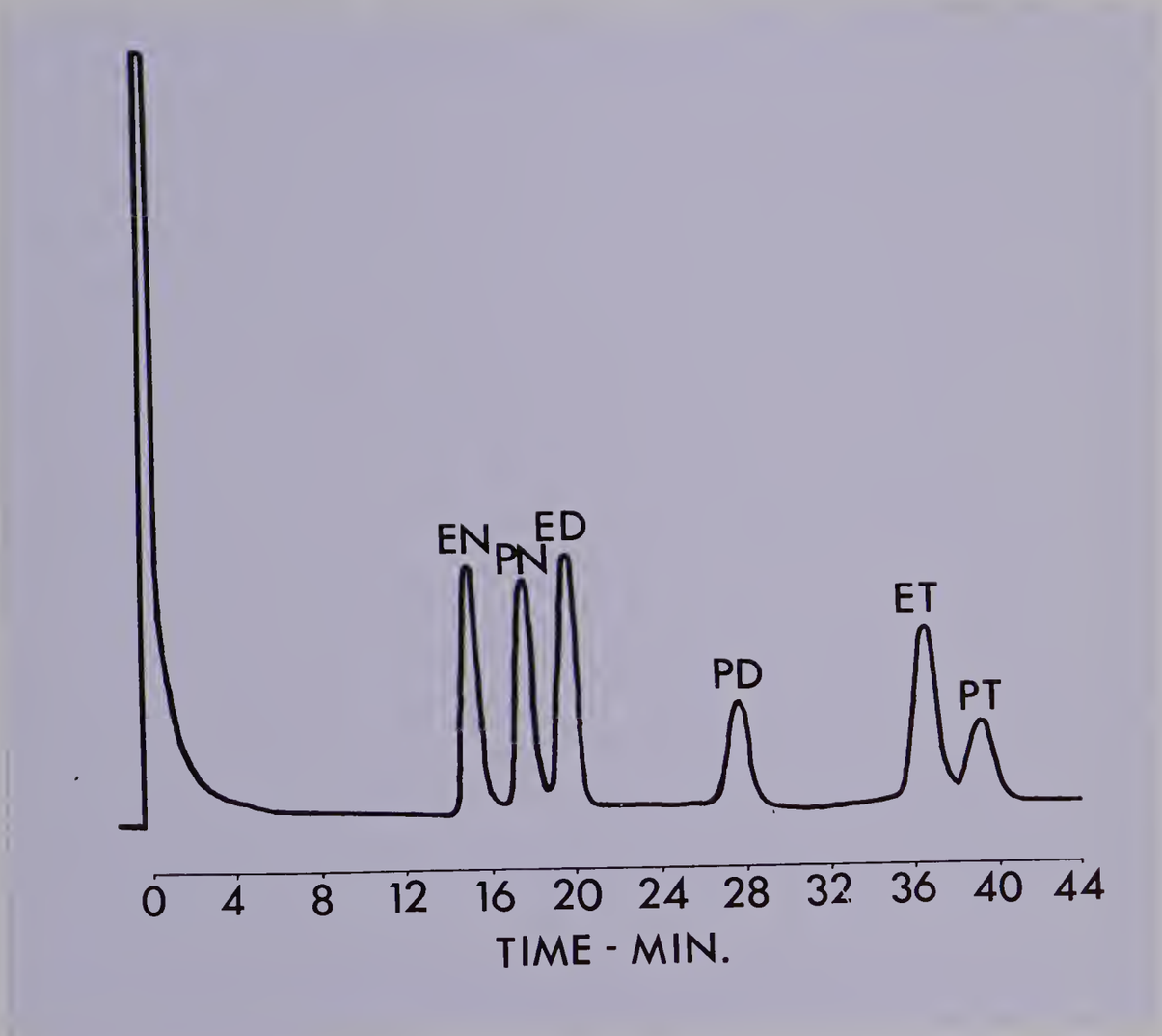

FIGURE XI. The GLC analysis of a mixture of estrogen and progesterone standards, using temperature programming as previously described. The compounds are the TMSi ether derivatives of estrone (EN), estradiol (ED), estriol (ET), pregnanolone (PN), pregnanediol (PD) and pregnanetriol (PT).

do not affect the separation process, but do alter the sensitivity of the detector, i.e., a hotter flame will cause a greater degree of ionization of the compounds and thus a greater detector response.

E. FINAL PROCEDURE DEVELOPED FOR THE DETERMINATION OF ESTRIOL, PREGNANEDIOL AND PREGNANOLONE IN PREGNANCY URINE.

It is generally agreed that the measurement of estriol, pregnanediol and pregnanolone may be of value in assessing fetal viability and placental function during pregnancy. Based upon the data obtained from the hydrolysis, derivatization and GLC studies, the following analytic method evolved for the determination of estriol, pregnanediol and pregnanolone in pregnancy urine.

The method offers several advantages over similar methods in that preliminary column or thin-layer chromatography(57), or separate hydrolysis of estriol and pregnanediol (58) or separation of estriol from pregnanediol and pregnanolone prior to GLC (8) is not required.

1. Methodology

A 50 ml. aliquot from a 24 hr. urine specimen was filtered and brought to pH 5.2 by the addition of glacial acetic acid or 10 N sodium hydroxide. Following this, 10 ml. of 0.2 M sodium acetate buffer (pH 5.2) and 0.5 ml. of Glusulase were added and the mixture incubated in a shaking water bath at 37^{o}C. for 18 - 24 hours.

The ether extractions of the hydrolyzed urine and the sodium bicarbonate washes were performed in the manner previously described. Since separation of estriol from pregnanediol and pregnanolone was not required, the sodium hydroxide extraction step could be omitted.

The ether extract of the steroids was evaporated to dryness by using a rotary evaporator *in vacuo* at 50°C. The residue was dissolved in small aliquots of acetone, transferred to a small volume flask and evaporated under rotary vacuum to *complete* dryness.

The dried extract was dissolved in 1 ml. of pyridine and 0.5 ml. of BSA and 25 µl. of TMCS were added. The mixture was allowed to stand at room temperature for ½ hr., then evaporated to dryness by using a rotary evaporator *in vacuo* at 65°C. The flask was tightly stoppered to protect the TMSi ether derivatives from moisture.

Programmed temperature GLC was carried out, using a column packed with 2% NGS on 80 - 100 mesh Chromosorb G. The operating conditions for GLC were as follows:

(1) Gas flow rates - as described previously.

(2) Initial temperatures - column 210°C., flash heater 270°C., flame detector 265°C.

(3) Temperature programming - Ten minutes after the sample was injected, the column temperature was raised at the rate of ½°C./minute.

The dried TMSi ether derivatives of the steroids were dissolved in 500 µl. of chloroform. Sample injections of 5 µl. were made, using a 10 µl. Hamilton syringe equipped with a Chaney adaptor.

TMSi ether derivatives of the steroid standards were prepared such that 5 µl. of the final chloroform solution contained 1 mcg. of each steroid derivative. Five mg. of each steroid was dissolved in 2 ml. of pyridine and 1.0 ml. of BSA and 0.05 ml of TMCS were added. After allowing ½ hr. for the derivative formation, the solution was evaporated to dryness in the usual manner. The residue was dissolved in 25 ml. of chloroform to produce a final concentration for each steroid of 1 mcg./5 µl. of solution. Although the standards in solution were stable for up to two months if protected from moisture, it was decided to avoid the risk of the standard solution coming into contact with moisture. To overcome this hazard, standard derivatives of the steroids were prepared each time an unknown was to be analyzed. This was accomplished by weighing out 5 mg. of each steroid, dissolving them in 25 ml. of acetone and transferring 1 ml. aliquots of the solution to small, teflon-capped vials. These were then taken down to dryness. When an analysis was to be performed, 1 ml. of acetone was added to a vial and 0.5 ml. of this solution pipetted into a small volume round bottom flask. This was taken down to

dryness, derivatized and injected in the previously described manner.

Comparison of standard peak areas with the corresponding unknown peak areas provided the basis for quantitation of the urinary steroids. The results were calculated by using the following formula:

$$\frac{\text{area of unknown}}{\text{area of standard}} \times \frac{\text{range-attenuation factor of unknown}}{\text{range-attenuation factor of standard}} \times \frac{\text{24 hr. urine volume (ml.)}}{500} = \text{mg. of steroid/24 hr.}$$

2. Significance of results

Figure XII demonstrates the separation characteristics of a standard mixture of the TMSi ether derivatives of estriol, pregnanediol and pregnanolone. Figure XIII shows the separation of the same derivatized steroids extracted from pregnancy urine by the above method. Usually, all the peaks emerged within 30 min. and were sufficiently separated to permit accurate quantitation.

To determine the recovery efficiency of the method, 0.1 mg. of each steroid was added to 50 ml. of male urine and run through the procedure. On the average, 85 - 95% of the added amount was recovered. Although addition of the conjugated steroids to the urine instead of the free steroids would have constituted a better recovery study, the difficulty of obtaining these steroids as conjugates prevented such a study. It was assumed hydrolysis was complete, as previously discussed.

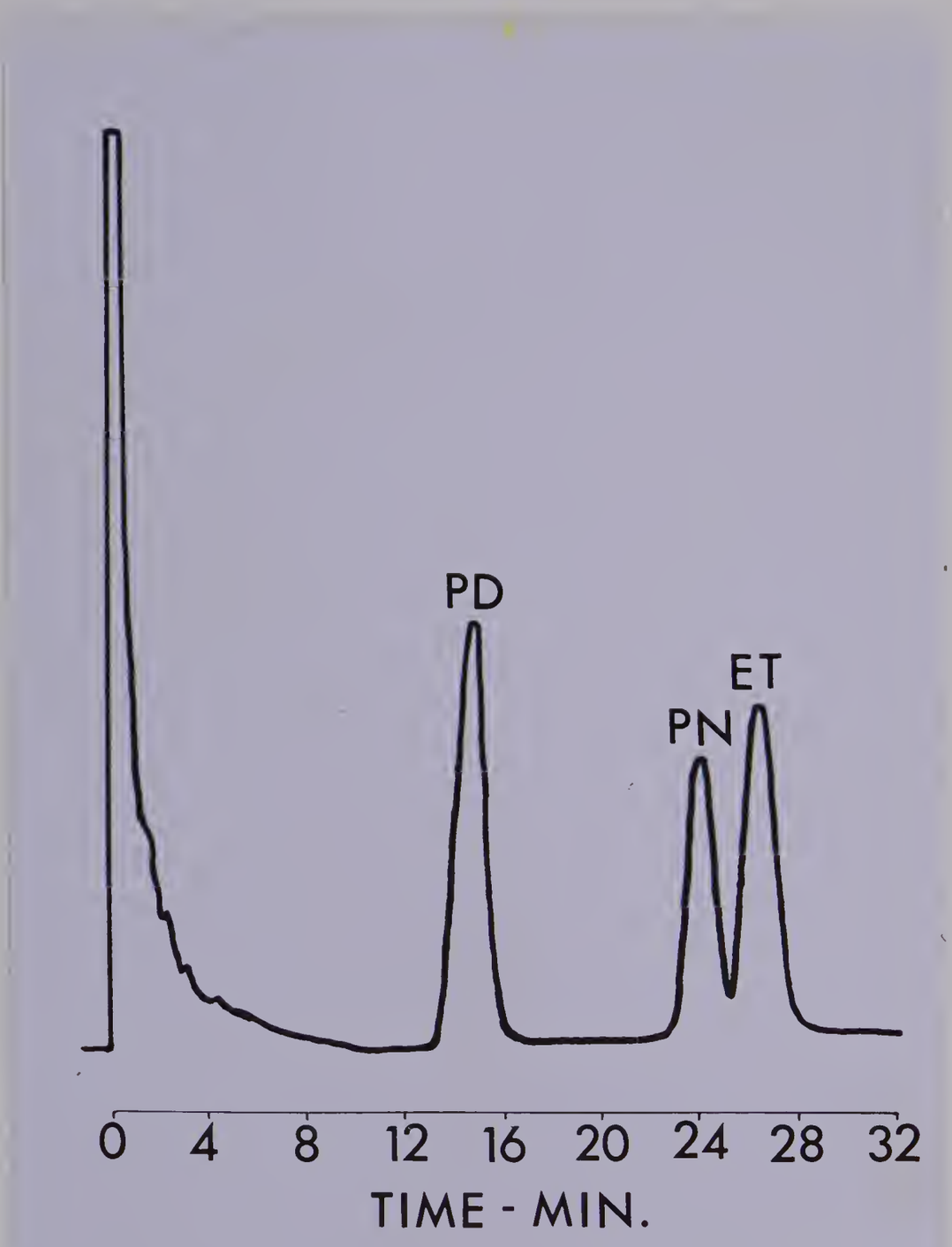

FIGURE XII. GLC analysis of standards. The compounds are the TMSi ether derivatives of estriol (ET), pregnanediol (PD) and pregnanolone (PN).

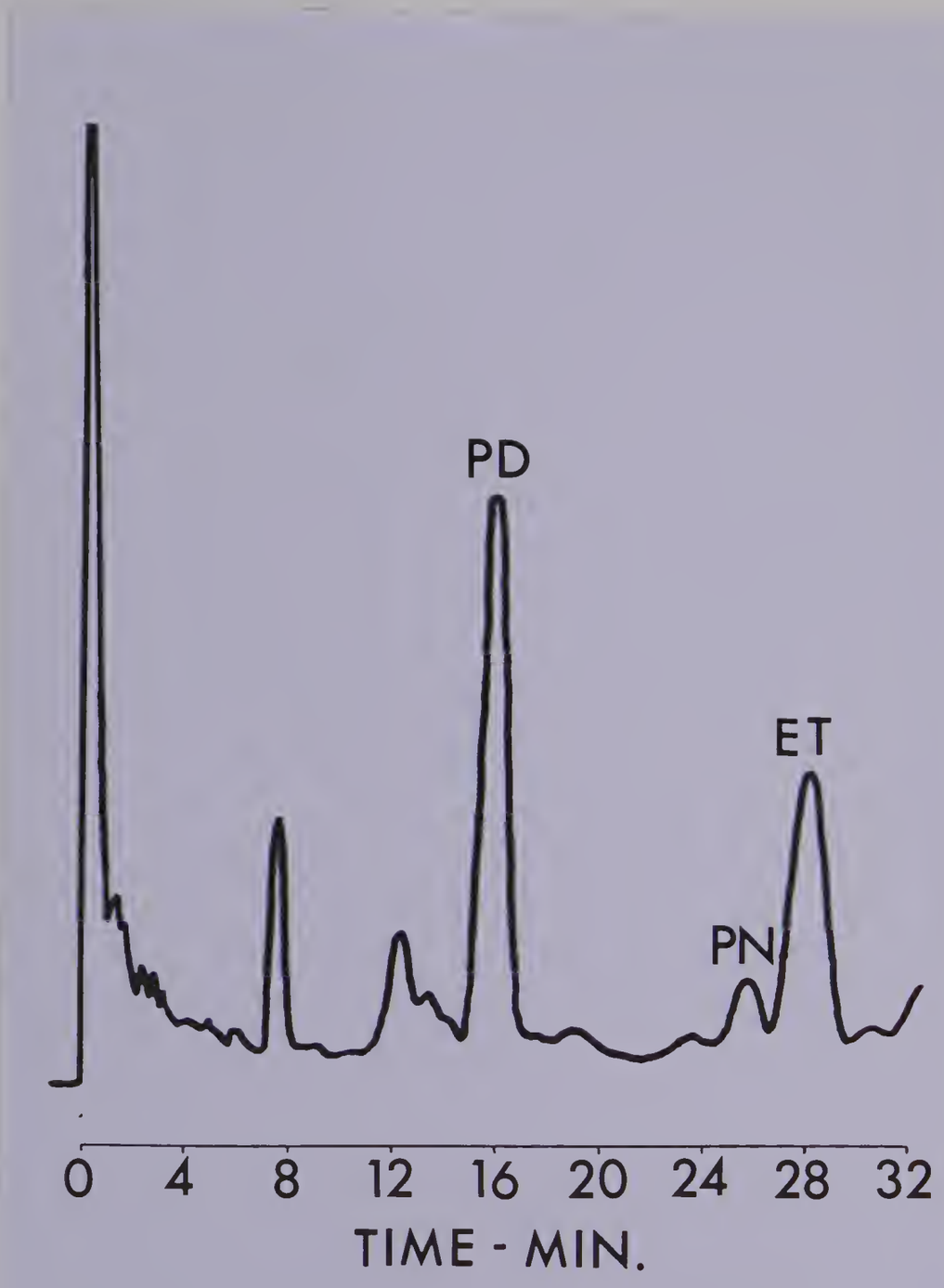

FIGURE XIII. GLC analysis of steroid fraction from pregnancy urine. The compounds are the TMSi ether derivatives of estriol (ET), pregnanediol (PD) and pregnanolone (PN).

Analysis of twenty different pregnancy urine specimens was carried out in duplicate and the precision of the method was calculated for the three metabolites concerned. The standard deviation (SD) was calculated according to the following formula:

$1\ SD = \pm \sqrt{\frac{\Sigma (d)^2}{2N}}$, where d is the difference between the paired (N) results.

Table III. shows the standard deviations and the range of various levels of estriol, pregnanediol and pregnanolone studied.

3. Diagnostic application

During the course of this study, two abnormal pregnancies were investigated. The usefulness of the above method in predicting the condition of the fetus and placenta is demonstrated.

TABLE III.

The precision (1 SD) attained for the analysis of estriol, pregnanediol and pregnanolone by the described method.

Steroid	Excretion level (mg./24 hr.)	Number of duplicate samples	Standard Deviation (mg./24 hr.)
Estriol	15 - 30	20	± 0.55
Pregnanediol	25 - 60	20	± 1.28
Pregnanolone	5 - 15	20	± 0.31

a. Placental function

CASE 1: Mrs. G.S., 22 years of age, para i, gravida ii, had a normal pregnancy in 1963. However, during her recent pregnancy (1967) she developed severe hypertension to which no cause could be attributed, other than the pregnancy itself. During hospitalization, her hypertension did not respond to medical treatment. In addition, the fetus had not grown appreciably during this period. It was decided to perform a Cesarean section at 35 weeks of gestation in the hope of saving the fetus and removing the mother's toxic condition. A 3 lb., 1 oz. female infant was delivered and, although premature, she did not exhibit any serious difficulties after being delivered.

Although the placenta appeared normal, it weighed only 300 grams. This is considerably smaller than a normal placenta which weighs between 500 and 600 gm. near term.

On two occasions, both within the week prior to delivery, urinary estriol, pregnanediol and pregnanolone levels were measured. The estriol was found to be 8.0 and 8.5 mg./24 hr., pregnanediol 12.2 and 12.8 mg./24 hr. and pregnanolone 2.5 and 2.2 mg./24 hr. on both occasions respectively.

Although the estriol levels are slightly below normal, they are not alarming and, in fact, agree with the extent of the fetus' maturity.

The abnormally low pregnanediol and pregnanolone values reflect the underdevelopment of the placenta. The small placenta was likely responsible for the mother's toxemia during pregnancy.

b. Fetal viability

CASE 2: Mrs. A.W., 25 years of age, para ii, gravida iv, had a history of Rh iso-immunization. Her first child was normal, the second required an exchange transfusion and the third, which was induced at 38 weeks gestation, died almost immediately following birth. In her recent pregnancy (1967), the Rh iso-immunization was already severe after only 28 weeks gestation. Amniocentesis showed the amniotic fluid contained 1.8 mgm.% bilirubin. An intra-uterine transfusion was successfully attempted, but a day later all fetal activity ceased. A week later, a 1 lb., 13 oz. stillborn was spontaneously delivered. The placenta weighed 550 grams.

The pathologist's report concluded that death of the fetus occurred intra-uterine, the cause being Rh incompatibility.

A 24 hr. urine specimen collected on the day before delivery was analyzed for estriol, pregnanediol and pregnanolone. Urinary estriol was only 3.7 mg./24 hr., indicating almost certain fetal death at that time, however, the pregnanediol level was found to be 45 mg./24 hr., indicating a normal, functioning placenta. Pregnanolone excretion was found to be 3.0 mg./24 hr., a slightly below normal value according to Larsen and Engstrom (8).

Thus, the findings of low estriol and normal pregnanediol levels in the urine confirmed what had actually occurred; intra-uterine fetal death, not due to placental dysfunction, but of fetal origin. As previously mentioned, urinary estriol and pregnanediol levels can be not used to determine the extent of Rh iso-immunization, except when the fetus has expired. Nevertheless, this example is typical of fetal dysfunction rather than placental.

4. Stability of stored specimens.

To adapt this method to routine laboratory use, a control of the method is necessary. Ideally, a urine specimen containing a known amount of the three steroids should be run through the procedure each time unknown specimens are analyzed. However, the stability of these steroids during storage, particularly pregnanediol, is questionable (59). In order to explore the possible use of stored urines for control purposes, the stability of estriol, pregnanediol and pregnanolone in pregnancy urine was studied.

Several pregnancy urine specimens were pooled, acidified with boric acid and divided into nine equal aliquots. The three steroids were immediately determined in one of of these aliquots, four were stored at $4^{o}C$. and the remaining four were stored at $-30^{o}C$. Every two weeks, one sample which was stored at $4^{o}C$. and one kept at

-30°C. were run, in duplicate, through the procedure and the amount of the three steroids in each sample estimated. Thus, the study covered a storage period of two months and two different temperatures.

The results of this study for estriol stored at two temperatures are shown in Figure XIV, the results for pregnanediol storage in Figure XV and for pregnanolone in Figure XVI. Examination of these data for all three steroids suggests that they are more stable at -30°C. However, as it can be seen from Figure XVI, pregnanolone is not particularly stable even at -30°C.

At the moment, it must be stated that there is no completely adequate quality control procedure for urinary steroids. Thus, when a low steroid value is obtained, assessment of the result with the obstetrician is mandatory.

F. DETERMINATION OF PREGNANEDIOL AND PREGNANETRIOL IN NON-PREGNANCY URINE

The value of knowing urinary pregnanediol and pregnanetriol levels in normal individuals and in various endocrine disease states has been already discussed.

Several GLC methods for quantitating pregnanediol in non-pregnancy urine have been published, but only a few have included the measurement of pregnanetriol (29, 34, 60). The advantage of determining both pregnanediol and pregnanetriol

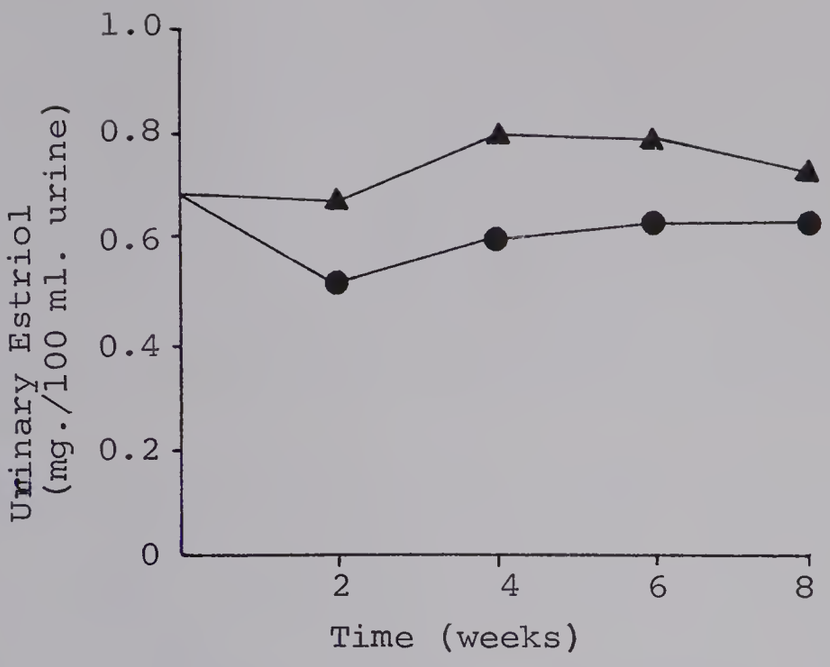

FIGURE XIV. The effect of storage on urinary estriol. (▲) stored at -30°C., (●) stored at 4°C.

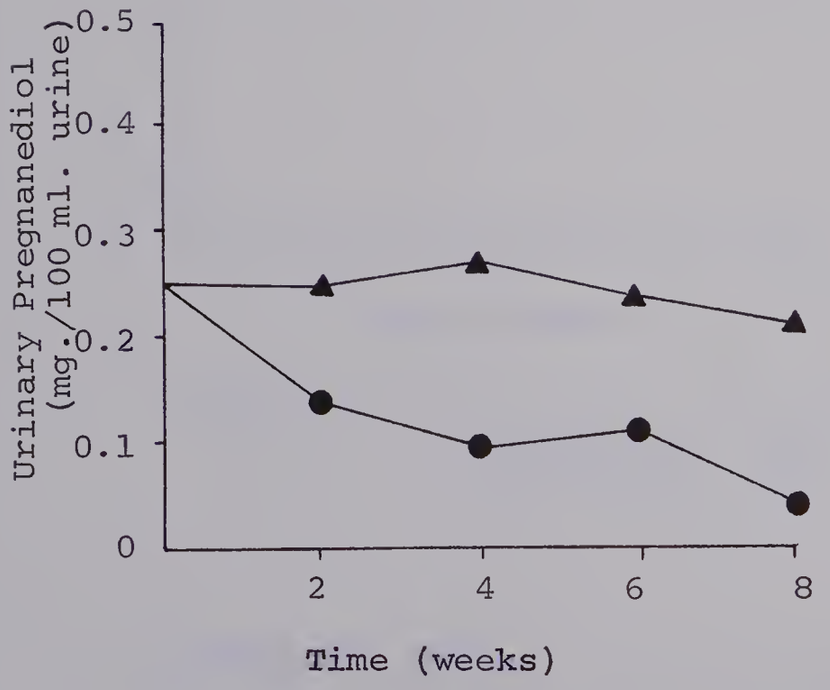

FIGURE XV. The effect of storage on urinary pregnanediol. (▲) stored at -30° C., (●) stored at 4°C.

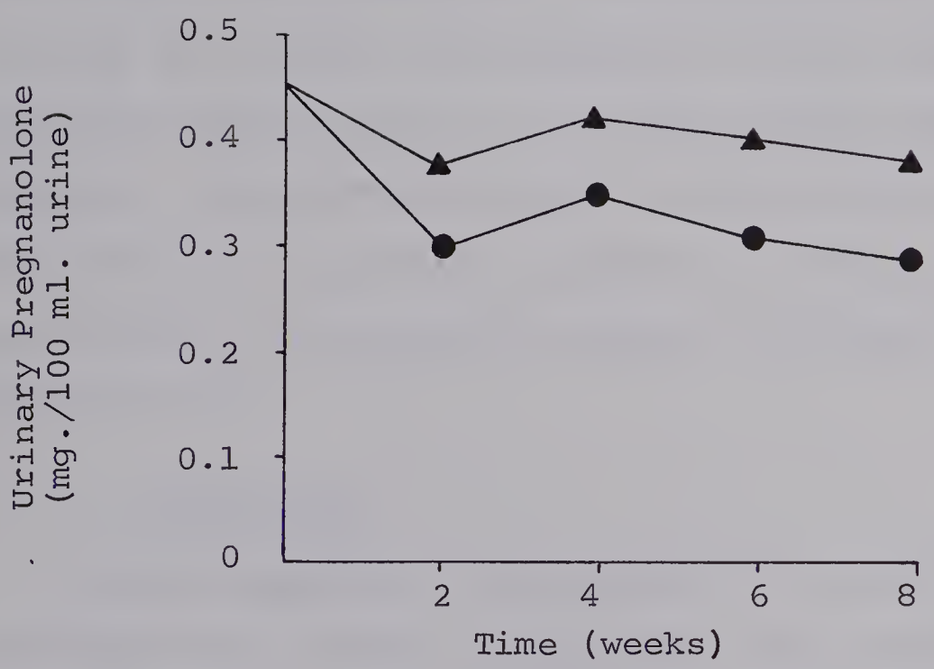

FIGURE XVI. The effect of storage on urinary pregnanolone. (▲) stored at -30°C., (●) stored at 4°C.

simultaneously is that both ovarian and adrenal function can be assessed together. A literature survey revealed only one report (34) of a procedure that did not require column chromatography prior to GLC analysis. For this reason, the following method for measuring urinary pregnanediol and pregnanetriol using GLC was developed.

The method to be described permits accurate quantitation of pregnanediol and pregnanetriol in non-pregnancy urine by GLC without the necessity of prior separation or extensive purification of the urine extract before GLC analysis. The method also shows some promise of being adaptable to the analysis of various 17-ketosteroids. Its rapidity and reliability makes it suitable for clinical laboratory use.

1. Methodology

Enzyme hydrolysis was carried out as outlined in the method for estriol, pregnanediol and pregnanolone in pregnancy urine.

The ether extractions, sodium bicarbonate wash and sodium hydroxide extractions were performed in the manner previously described. If estriol was to be determined on the urine specimen, the sodium hydroxide extraction was saved.

The ether phase containing the progesterones was dried over anhydrous sodium sulfate and then evaporated

to dryness using a rotary evaporator in vacuo at 50°C. The residue was dissolved in small aliquots of acetone, transferred to a small volume round bottom flask and then evaporated to complete dryness in the same manner as above.

The TMSi ether derivatives were formed as previously described in the section on urinary estriol, pregnanediol and pregnanolone.

Programmed temperature GLC was carried out using the same type of column and operating conditions as described for GLC analysis of estriol, pregnanediol and pregnanolone.

The dried TMSi ether derivatives of pregnanediol and pregnanetriol were dissolved in 200 µl. of chloroform. Sample injections of 5 µl. were made using a 10 µl. Hamilton syringe equipped with a Chaney adaptor.

The method for preparing pregnanediol and pregnanetriol standards was the same as the second method described for preparing estriol, pregnanediol and pregnanolone standards.

The results were calculated by using the following formula:

$$\frac{\text{area of unknown}}{\text{area of standard}} \times \frac{\text{range-attenuation factor of unknown}}{\text{range-attenuation factor of standard}} \times \frac{\text{24 hr. urine volume (ml.)}}{1{,}250} = \text{mg. of steroid/ 24 hr.}$$

2. Significance of results

Figure XVII demonstrates the separation characteristics of a standard mixture of the TMSi ether derivatives of pregnanediol and pregnanetriol. Figure XVIII shows the separation of the same derivatized steroids extracted from non-pregnancy urine by the method described above. GLC was usually complete after 45 minutes. Pregnanediol and pregnanetriol derivatives in the urine specimen were identified by comparing their retention times to the retention times of pregnanediol and pregnanetriol derivatives in the standard mixture. Furthur identification was made by injecting a urine sample before and after the addition of derivatized pregnanediol and pregnanetriol from the standard.

Although other peaks present in the chromatograms were not identified, many of these were probably due to the presence of some of the 17-ketosteroids (34). A slight modification of the GLC operating conditions and identification of these peaks would probably allow quantitation of some of the 17-ketosteroids.

To determine the recovery efficiency of the method, 60 mcg. each of pregnanediol and pregnanetriol were added to one of two aliquots of the same urine specimen. Both specimens were run through the procedure and the individual differences between pregnanediol and pregnanetriol in both urines was calculated. The differences (in mcg.)

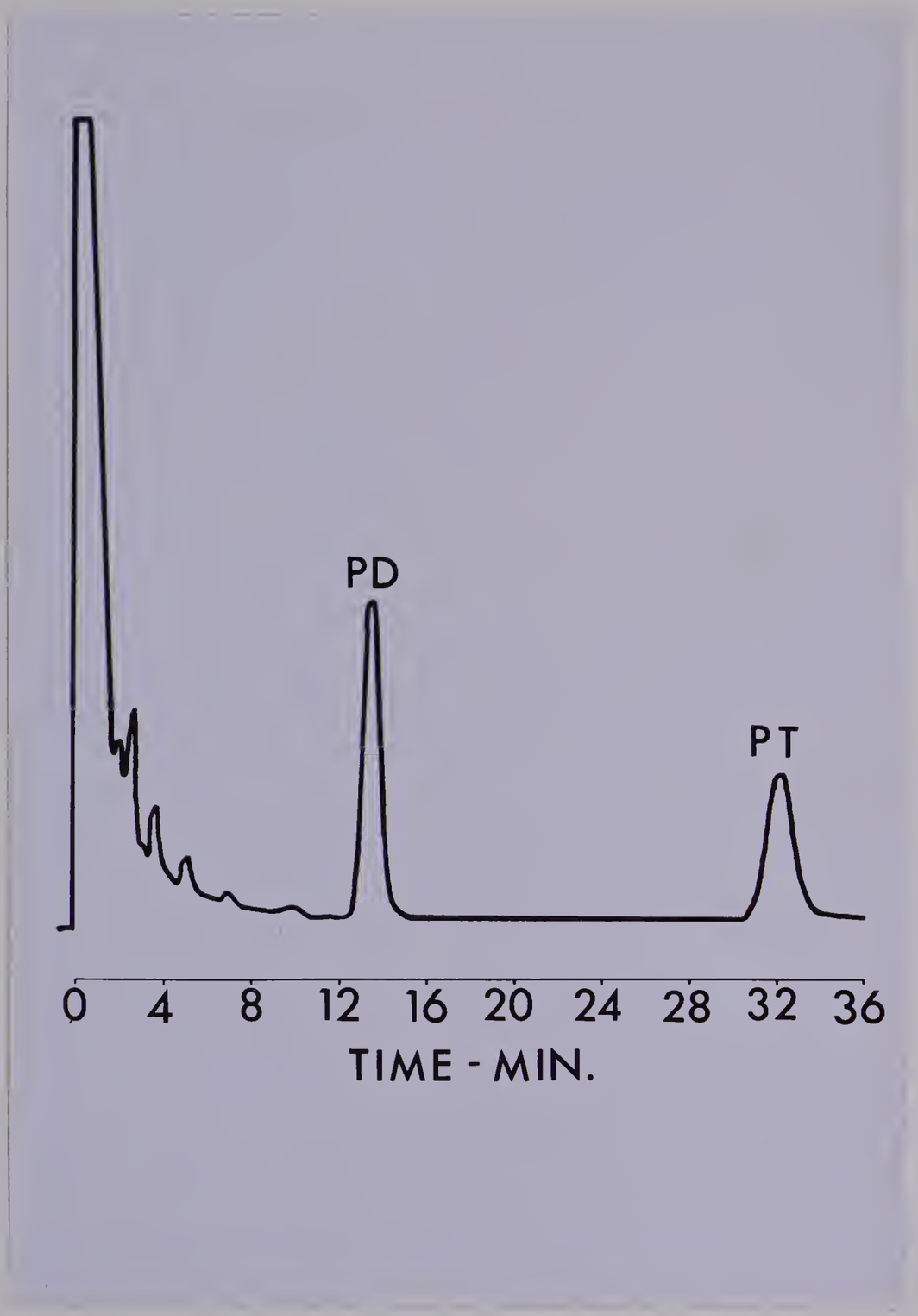

FIGURE XVII. The GLC analysis of a standard mixture of the TMSi ether derivatives of pregnanediol (PD) and pregnanetriol (PT).

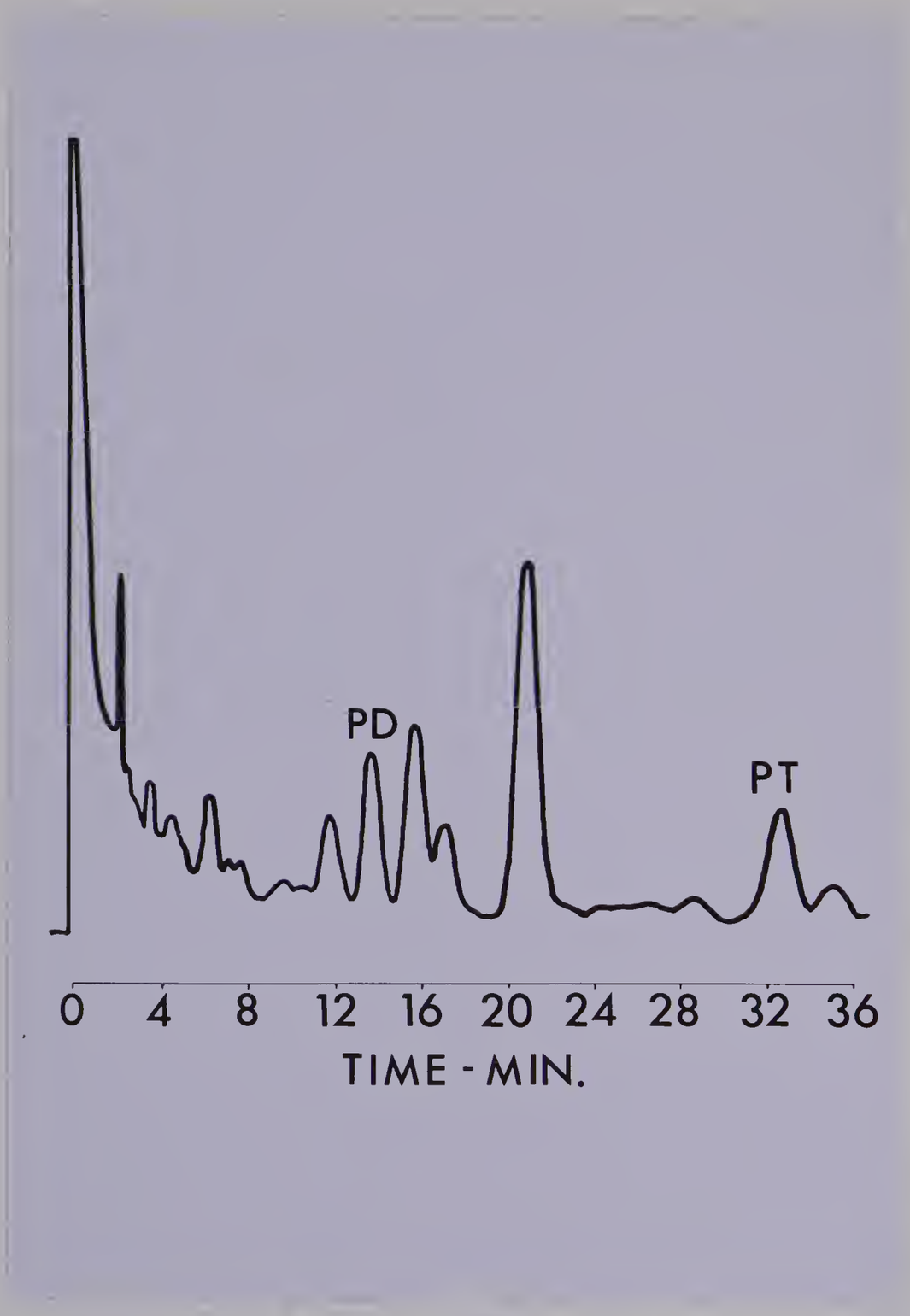

FIGURE XVIII. The GLC analysis of the TMSi ether derivatives of pregnanediol (PD) and pregnanetriol (PT) extracted from non-pregnancy urine.

were expressed as a percentage of the original amount of each steroid added. On the average, 70 - 80% of each added steroid was recovered.

The analysis of thirty-five different non-pregnancy urine specimens was carried out in duplicate and the SD was calculated for both pregnanediol and pregnanetriol. Table IV shows the standard deviations and the range of various levels of pregnanediol and pregnanetriol in which the analyses were made.

3. Two examples of diagnostic application

a. Menstrual cycle variations

A normal, 24 year old female collected 24 hr. urine specimens every second day during a complete menstrual cycle. On the day each sample was received, the urine was set up for enzyme hydrolysis and the extractions and

TABLE IV.

The precision (1 SD) attained for analysis of pregnanediol and pregnanetriol by the described method.

Steroid	Excretion level (mg./24 hr.)	Number of duplicate samples	Standard Deviation (mg./24 hr.)
Pregnanediol	0.20 - 4.80	35	± 0.02
Pregnanetriol	0.40 - 1.50	35	± 0.01

GLC performed next day. The creatinine content of each specimen was determined to ascertain whether or not the collection had been properly timed.

Figure XIX shows the urinary pregnanediol output during this study. Ovulation probably occurred around the eleventh day of her cycle, since it usually takes a day or two for the corpus luteum to begin production of progesterone. Degeneration of the corpus luteum occurred on about the twenty-second day of the cycle, as indicated by the decreased pregnanediol output following that day. Following this, the pregnanediol output rapidly decreased and menstruation began on the twenty-seventh day. Little variation of urinary pregnanetriol output occurred during this cycle study.

The urines of several other normal, ovulating females were studied, but not in such detail. Twenty-four hr. urine specimens were collected weekly over a period of several menstrual cycles. Figure XX shows the urinary pregnanediol output of one subject during three consecutive menstrual cycles. Figure XX also shows the urinary pregnanetriol output during the same period and demonstrates that fluctuations of the pregnanetriol level occurred. This confirmed the observations of Pickett *et al* (61), who have suggested that the ovary is possibly an additional source of pregnanetriol.

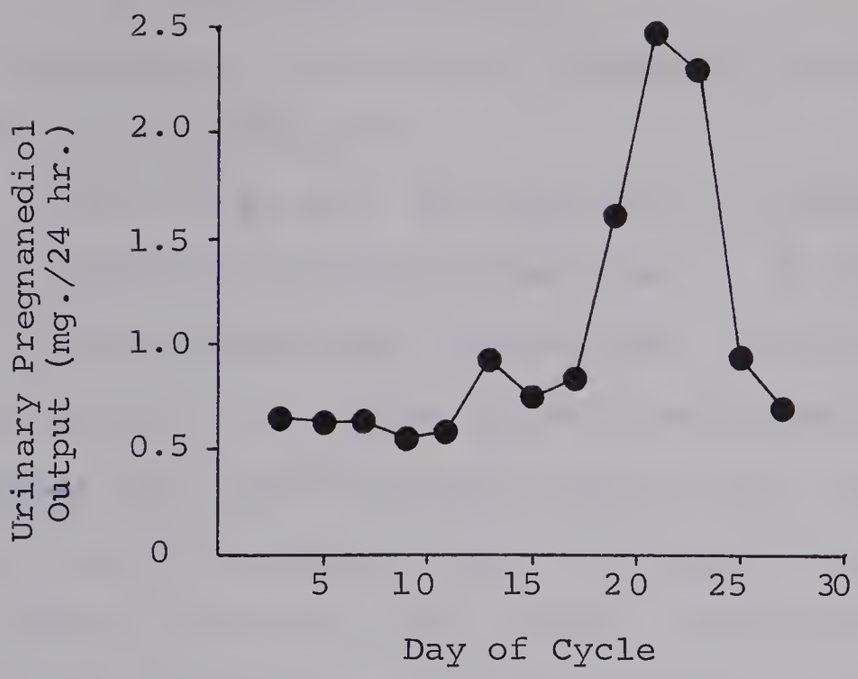

FIGURE XIX. The urinary excretion of pregnanediol throughout a single menstrual cycle.

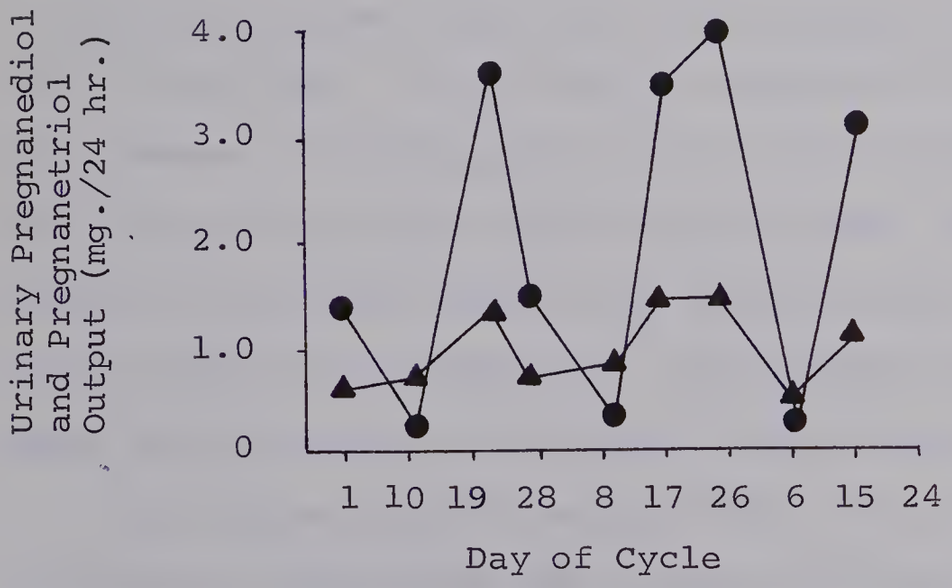

FIGURE XX. The urinary excretion of pregnanediol (●), and pregnanetriol (▲) throughout three consecutive menstrual cycles. The lengths of the cycles were 28, 26 and 24 days, respectively.

b. In adrenal disease

One patient affected with congenital adrenogenital syndrome was investigated.

In 1950, Miss C.W. was diagnosed as having adrenogenital syndrome (probably congenital). At that time, her 17-ketosteroids level was markedly elevated (up to 117 mg./24 hr.). Cortisone acetate replacement therapy decreased the 17-ketosteroids level to 35 - 40 mg./24 hour. During the next eighteen years, she gradually escaped from medical follow-up and control. Consequently, she was not on corticoid replacement therapy for several years.

In 1968 she was re-admitted to hospital with complaints of a persistent cough and general weakness. As before, 17-ketosteroids were very high and cortisone therapy was re-installed. A bone marrow examination revealed the presence of widespread typical adenocarcinoma cells, but the site of origin could not be stated. She did not respond to any drug therapy and expired within two weeks of her admission to hospital. The immediate cause of death was attributed to bilateral bronchiopneumonia.

The autopsy report concluded that the primary cause of death was adenocarcinoma of the left lung with secondary metastases. Adrenal hyperplasia was noted, thus confirming her long-standing adrenogenital syndrome.

Urinary pregnanediol and pregnanetriol levels were measured by the method previously described. The pregnanediol

level was 3.5 mg./24 hours. This was not abnormal since she was still in her pre-menopausal life (36 years of age). The pregnanetriol level was 76.0 mg./24 hr., tremendously elevated. Due to her long-standing case of adrenogenital syndrome, it is probably that the elevated urinary pregnanetriol level resulted from this adrenal dysfunction rather than due to the presence of the tumor. However, the remote possibility of the tumor producing the increased levels of pregnanetriol can not be denied. The latter possibility could have been ruled out if analysis for pregnanetriol in the tumor tissue had been performed.

G. DETERMINATION OF ESTRIOL IN NON-PREGNANCY URINE

The value of determing urinary estrogens in non-pregnancy urine with respect to ovarian function is well established. Whether or not estrone and estradiol need be determined in addition to estriol is doubtful. No known disease has shown one of the "classical estrogens" to be elevated without a corresponding rise in the other two. The determination of estriol alone should reflect estrogen metabolism as a whole, since it is the final product of estrogen metabolism. Thus, it seems likely that determination of estrone, estradiol and estriol simultaneously is unnecessary.

Several GLC methods for determing estrogens in non-

pregnancy urine have been recently published (62-64). All methods involve at least two preliminary purifications of the urine extract by thin-layer chromatography, column chromatography or benzene-water partition. The formation of at least two different types of estrogen derivatives during the purification steps was also required. Needless to say, these methods are far too lengthy to serve the needs of many clinical laboratories. The need for a simplified and accurate GLC method for measuring estriol in non-pregnancy urine was responsible for this investigation.

The separation of estriol from many interfering compounds in the urine was attempted by different types of programmed temperature GLC. Although separation of many compounds was enhanced, estriol quantitation was not possible. The use of column chromatography (65) prior to programmed temperature GLC resulted in the removal of some contaminants, but accurate quantitation of estriol was difficult. Thin-layer chromatography was investigated using several different solvent systems. Detection of estriol on the plates with 2',7' Dichlorofluorescein(DCF) was difficult with all systems since various constituents in the urine extract obscurred visualization of estriol. Applying the extract, which represented 250 ml. of urine, presented furthur difficulties. When the final extract was dissolved in 100 µl. of acetone, a tarry solution often

resulted and when this was applied to the plate, it was difficult to obtain a homogeneous "spot", i.e., all of the extract being in contact with the silicic acid. This was overcome to some extent by "streaking" the sample across the plate. However, when this was done, detection of estriol by DCF was even worse, since the estriol was spread over a large area and gave no detectable fluorescence.

Benzene-water partition (66) of estriol from other urinary constituents, prior to programmed temperature GLC was attempted. Although the removal of many interfering compounds was accomplished, reproducibility and recovery of added estriol was very eradic.

Most purification steps occur before derivatization of estriol. Only TLC of derivatized estriol has been investigated (57,62). The advantage to be gained by purification of estriol after derivatization lies in the change of the properties of estriol upon derivatization, i.e., from a relatively polar steroid to a non-polar steroid.

The possibility of a single solvent extraction of derivatized estriol from the undesirable urinary components was investigated. It was found that n-hexane quantitatively extracted derivatized estriol without extracting most other urinary constituents.

The method to be described permits determination of

estriol in non-pregnancy urine. It represents a significant simplification of other methods used for the analysis of non-pregnancy urinary estriol, whilst maintaining their precision and efficiency.

1. Methodology

A portion of a 24 hr. urine specimen was concentrated five fold by evaporation under rotary vacuum at 40°C. The concentrated specimen was cooled, then filtered and set up for enzyme hydrolysis in the same manner described for the simultaneous determination of estriol, pregnanediol and pregnanolone in pregnancy urine.

The hydrolyzed urine was extracted and purified according to the procedure described under EXTRACTION PROCEDURE (section B.),i.e., ether extraction, sodium bicarbonate wash, sodium hydroxide extraction, acidification and re-extraction into ether.

The TMSi ether derivative of estriol was formed as previously described for the derivatization of estriol, pregnanediol and pregnanolone in pregnancy urine. After the derivatives had been formed (½ hr.), the solution was evaporated under rotary vacuum at 65°C. to dryness. At this stage, three successive 5 ml. n-hexane extractions of the derivatized urine extract were performed. It was found that incomplete extraction of estriol resulted, probably due to incomplete contact of the n-hexane with the urine extract,i.e., only the surface of the extract

was in contact with the n-hexane. This was remedied by dissolving the derivatized extract in 1 ml. of chloroform and then drying this solution onto a circular piece of filter paper (Whatman #1) under rotary vacuum at 65°C. Prior washing of the filter paper by acetone prevented introduction of other contaminants from the filter paper. The TMSi ether derivative of estriol was then extracted by three successive 5 ml. aliquots of n-hexane and the n-hexane then transferred to a small volume round bottom flask and evaporated to dryness by the usual method. When programmed temperature GLC was to be performed, the n-hexane extract was dissolved in 200 µl. of chloroform. Injections of 5 µl. were made using a 10 µl. Hamilton syringe equipped with a Chaney adaptor.

A 2% NGS liquid phase on 80 - 100 mesh Chromosorb G. was packed in a 6 ft. glass U-column. The conditions for GLC were as follows:

(1) Gas flow rates - as previously described.

(2) Initial temperatures - column 220°C., flash heater 270°C., flame detector 265°C.

(3) Temperature programming - Ten min. after the sample was injected, the column temperature was raised at the rate of ½°C./minute.

The method for preparation of estriol standard was the same as the second method described for preparing

estriol, pregnanediol and pregnanolone standards.

The results were calculated by using the following formula:

$$\frac{\text{area of unknown}}{\text{area of standard}} \times \frac{\text{range-attenuation factor of unknown}}{\text{range-attenuation factor of standard}} \times \frac{\text{24 hr. urine volume (ml.)}}{6.25} = \text{mcg. estriol/24 hr.}$$

2. Significance of results

Figure XXI shows the separation characteristics of a urine extract without the n-hexane extraction step prior to GLC analysis. Figure XXII shows the separation characteristics of the same non-pregnancy urine extracted with n-hexane following derivatization. The advantage of the n-hexane extraction is clearly illustrated by the fact that the sensitivity factor of the electrometer in Figure XXII could have been raised two to four fold, but a similar increase in sensitivity for Figure XXI would have placed the baseline and peak of the chromatogram off-scale.

The use of 3% OV-1 instead of 2% NGS was investigated. The non-selective qualities of OV-1 did not give as good a baseline for quantitation as did NGS. However, the OV-1 liquid phase was useful for confirming the presence of derivatized estriol in the urine extract and, in fact, confirming that the peak measured when using the NGS column was indeed estriol. Furthur identification of the

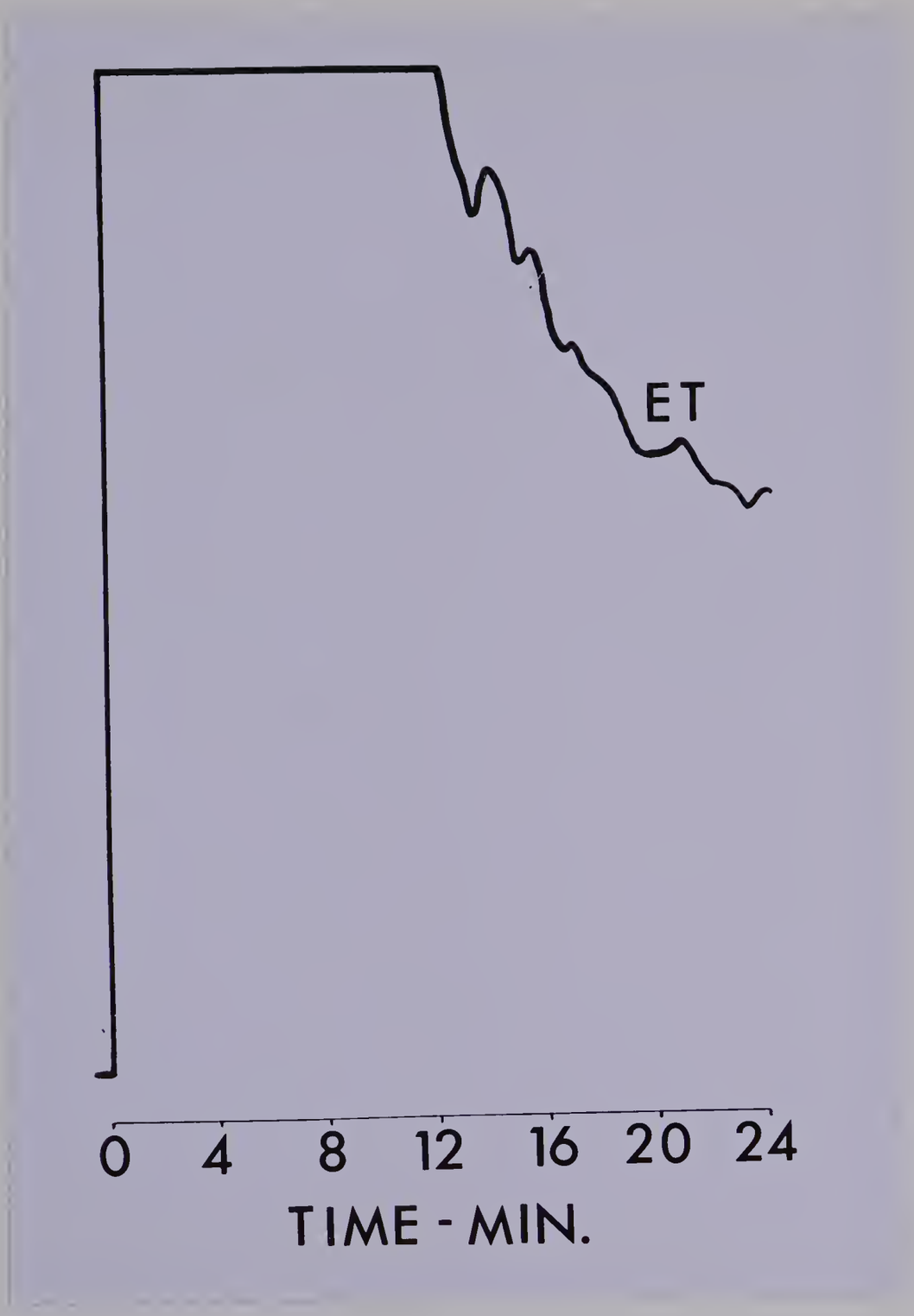

FIGURE XXI. The GLC analysis of a non-pregnancy urine extract containing estriol, prior to n-hexane extraction. Estriol (ET) is shown as a TMSi ether derivative.

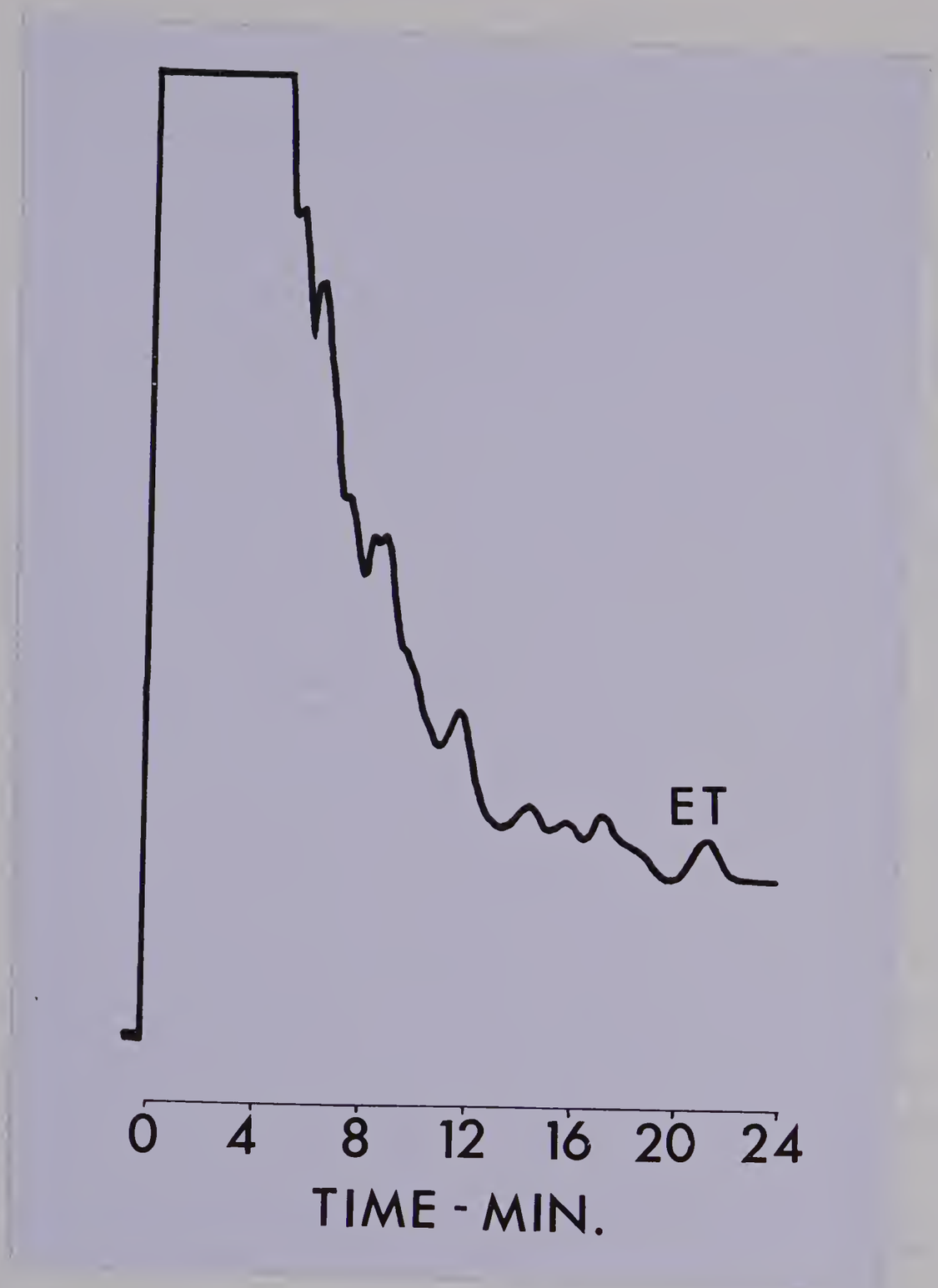

FIGURE XXII. The GLC analysis of a non-pregnancy urine extract containing estriol, after n-hexane extraction of the TMSi ether derivative of estriol (ET).

estriol peak was made by injecting a urine sample before and after the addition of derivatized estriol from the standard.

To determine the recovery efficiency of the method, 10 mcg. of estriol was added to one of two aliquots of the same urine specimen. Both aliquots were subjected to the described procedure and the difference in the amount of estriol in both samples was calculated and expressed as a percentage of the original amount of estriol added. On the average, greater than 90% of the added estriol was recovered.

The analysis of fifteen different non-pregnancy urine specimens was carried out in duplicate. The precision (1 SD) of the method was $\pm$ 1.9 mcg. estriol/24 hr. at the 5 to 50 mcg./24 hr. level.

The clinical usefulness of estriol measurements in various ovarian and menstrual cycle disorders is well-established in the review article by Brown (2). Thus, clinical problems related to estrogen production were not studied by the GLC method developed here. However, the fact that a significantly shorter method has been developed in comparison to previous lengthy procedures, which require up to three days for results to be obtained, should furthur stimulate the study of estriol excretion in clinical problems which involve abnormal estrogen metabolism.

III. CONCLUSIONS

(1) A reproducible GLC method for the simultaneous analysis of estriol, pregnanediol and pregnanolone in pregnancy urine was developed. Thus, the obstetrician will be able to evaluate both fetal viability and placental function when these measurements are simultaneously available.

(2) A reproducible GLC method for the simultaneous determination of pregnanediol and pregnanetriol in non-pregnancy urine was developed. By analyzing both progesterone metabolites together, information on ovarian and adrenal function was made possible. The application of this method involved a detailed study of pregnanediol excretion during a normal menstrual cycle and random pregnanediol and pregnanetriol determinations during several consecutive menstrual cycles. The usefulness of this method in determining pregnanetriol was demonstrated in a documentated case of adrenogenital syndrome.

(3) A reproducible GLC method for determining estriol in non-pregnancy urine was developed. In contrast to all previously published GLC methods for non-pregnancy estriol, the developed method involved a simple organic solvent extraction of the derivatized urinary estriol immediately prior to GLC analysis. All other methods require more extensive and elaborate preliminary purification steps in order to achieve accurate quantitation of estriol. Although no clinical studies involving the

use of this method are reported, the value of urinary estriol measurements in the diagnosis of various ovarian and menstrual cycle disorders is well-established.

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